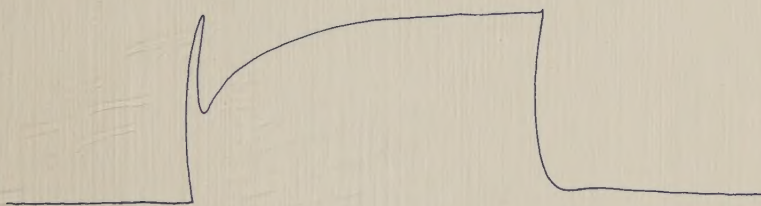


On the Role of Calcium in Depolarization-Induced and Receptor-Mediated Contractions in Isolated Cerebral and Peripheral Arteries



by

Edward D. Högestätt

LUND 1984



From the Department of Clinical Pharmacology
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A small step for mankind
a giant leap for me

The present thesis is based on the following studies, which in the text will be referred to by their Roman numerals:

- I** Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. Högestätt, E.D., Andersson, K.-E. & Edvinsson, L., *Acta Physiol Scand* 117:49-61, 1983.
- II** Mechanisms behind the biphasic contractile response to potassium depolarization in isolated rat cerebral arteries. Högestätt, E.D. & Andersson, K.-E., *J Pharmacol Exp Ther*, 228:187-195, 1984.
- III** Effects of nifedipine on potassium-induced contraction and noradrenaline release in cerebral and extracranial arteries from rabbit. Högestätt, E.D., Andersson, K.-E. & Edvinsson, L., *Acta Physiol Scand* 114:283-296, 1983.
- IV** Characterization of two different calcium entry pathways in small mesenteric arteries from rat. Högestätt, E.D., *Acta Physiol. Scand.* Accepted for publication.
- V** On the postjunctional α -adrenoceptors in rat cerebral and mesenteric arteries. Högestätt, E.D. & Andersson, K.-E. Submitted for publication.
- VI** Influence of extracellular calcium and nifedipine on α_1 - and α_2 -adrenoceptor mediated contractile responses in isolated rat and cat cerebral and mesenteric arteries. Skärby, T., Högestätt, E.D. & Andersson, K.-E. Submitted for publication.

Abbreviations: VSM, vascular smooth muscle; NA, noradrenaline; POC, potential-operated Ca^{2+} channel; ROC, receptor-operated Ca^{2+} channel; 6-OHDA, 6-hydroxydopamine; EGTA, ethylene glycol bis (β -aminoethyl ether) $\text{N,N'$ -tetraacetic acid.

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INTRODUCTION

Role of Ca^{2+} in muscle activation

The essential role of Ca^{2+} in muscle contraction was recognized more than a century ago by Sidney Ringer in his pioneering work on the influence of different constituents of the blood on the contractile activity of the isolated frog heart (Ringer 1883). Since then, our understanding of the regulation of muscle contraction has grown considerably. It is now generally accepted that the myoplasmic (free) Ca^{2+} concentration is the prime regulator of the contraction-relaxation cycle in all types of muscle, although the regulatory mechanisms at the level of the contractile proteins appear to be markedly different in striated and smooth muscle (Ebashi 1980, Stull & Sanford 1981, Webb & Bohr 1981).

In relaxed muscle, whatever type, the myoplasmic Ca^{2+} concentration is maintained below 10^{-7} M (Ebashi 1980, Stull & Sanford 1981). The highly asymmetric distribution of Ca^{2+} across the cell membrane (10^{-3} M outside versus 10^{-7} M inside) is achieved by mechanisms which reduce the Ca^{2+} concentration in the myoplasm, such as Ca^{2+} extrusion and intracellular Ca^{2+} binding and/or uptake (Somlyo & Somlyo 1968, Bolton 1979, Adam & Schwartz 1981, Webb & Bohr 1981). Following stimulation of the muscle cells, the myoplasmic Ca^{2+} concentration is increased, leading to activation of actomyosin and contraction (Ebashi 1980, Stull & Sanford 1981). Principally, this can be achieved either by influx of Ca^{2+} from the extracellular medium or by release of Ca^{2+} from intracellular stores.

Contribution of different Ca^{2+} pools

In vertebrate skeletal muscle, the contractile activity depends almost entirely on release of Ca^{2+} from the sarcoplasmic reticulum (Andersson 1978, Adam and Schwartz 1981). Relaxation is effected by an active reuptake of Ca^{2+} into the sarcoplasmic reticulum, and the ion may subsequently be re-utilized for contraction (Andersson 1978, Adam & Schwartz 1981). However, in

vascular smooth muscle (VSM), the role of different Ca^{2+} sources in the contractile activation and the cellular mechanisms regulating the intracellular Ca^{2+} concentration are less well understood.

Much information on the excitation-contraction coupling process in VSM has been gained from studies of isolated blood vessel preparations. While studying the effect of adrenaline on the isolated rabbit aorta, Bohr (1963) observed that the contraction induced by this agent could be resolved into an initial fast and an ensuing slow (tonic) component, differentially affected by the external Ca^{2+} concentration. Van Breemen (1972) and Deth and van Breemen (1974, 1977), using the sympathetic neurotransmitter noradrenaline (NA) to stimulate contraction, reported similar observations and provided strong evidence that the initial fast component and the ensuing slow component of the NA-induced contraction were associated with release of Ca^{2+} from intracellular stores and influx of Ca^{2+} from the extracellular medium, respectively. Subsequent studies have indicated that the relative contribution of Ca^{2+} influx and intracellular Ca^{2+} release to the contractile activation varies not only between VSM from different species and regions, but also with the mode of excitation (Bolton 1979, Webb & Bohr 1981). Notably, the releasable intracellular Ca^{2+} depots in VSM, in contrast to those in skeletal muscle, appear to be relatively limited and/or easily depleted in Ca^{2+} -free medium, since repetitive or sustained contractions usually cannot be induced in the absence of extracellular Ca^{2+} (Bolton 1979, Webb & Bohr 1981). The cellular localization of the releasable Ca^{2+} pool in VSM has not been definitely established, although the sarcoplasmic reticulum and the inner aspect of the cell membrane have been proposed as likely candidates (Devine et. al 1972, Bolton 1979, Webb & Bohr 1981).

Electro- and pharmacomechanical coupling

In vertebrate skeletal and cardiac muscle, the action potential normally triggers the contractile response (Andersson 1978, Adam & Schwartz 1980). However, electrophysiological studies of VSM have indicated that the electrical events accompanying the

mechanical response vary considerably between different VSM preparations and modes of excitation. Based on the type of electrical response associated with the contractile activity, VSM may be divided into spike generating VSM (e.g. the rat portal vein) and non-spike generating VSM (e.g. the rabbit pulmonary artery), the latter responding to contracting agents with graded depolarization (Somlyo & Somlyo 1968, Johansson & Somlyo 1980). However, in some VSM preparations (e.g. the rabbit ear artery), certain agonists can induce contraction in the absence of membrane depolarization (Droogmans et al. 1977). This mode of activation has been termed pharmacomechanical coupling (Somlyo & Somlyo 1968).

A classical way to study the relationship between membrane depolarization and contraction is to expose the preparation to solutions with high K^+ concentrations. In isolated frog skeletal muscle fibres, exposure to high K^+ depolarizing medium induces a transient contracture (Hodgkin & Horowicz 1960). However, most smooth muscle tissues respond to K^+ with sustained contractions, which usually can be divided into two or more components (Bolton 1979). The mechanisms behind the different components are poorly understood and seem to vary from preparation to preparation. One thing that should be kept in mind is that high K^+ solutions may also cause neuronal transmitter release, leading to stimulation of receptors at the muscle cell membrane and modulation of the mechanical response (see, e.g., Vanhoutte & Verbeuren 1976). Thus, if the depolarizing effect of K^+ on the VSM cells is to be studied, a neurogenic contribution to the K^+ response has to be excluded or sufficiently eliminated.

Ca^{2+} channels

Extracellular Ca^{2+} has been suggested to enter the VSM cells in several ways: (1) via specific membrane channels for Ca^{2+} , (2) in exchange for Na^+ by a hypothetical Na^+-Ca^{2+} exchange mechanism and (3) by a less well defined "passive leak" of the ion across the cell membrane, accounting for the Ca^{2+} uptake in the resting muscle (Bolton 1979, van Breemen et al. 1979). Bolton (1979) proposed the existence of two different types of membrane channel for Ca^{2+} , termed potential- and receptor-operated channels (POCs

and ROCs), in smooth muscle. According to his definition, the POC is an ion channel population that opens when the potential across the membrane is reduced, e.g., after exposure to K^+ -rich medium. This channel is considered to correspond to the "slow channel" in cardiac muscle. ROCs, on the other hand, were suggested to be primarily controlled or operated by receptors for stimulant substances. The concept of two types of Ca^{2+} channel has proved useful to explain the actions of various drugs on VSM. Furthermore, Ca^{2+} influx via ROCs, either alone or in combination with intracellular Ca^{2+} release, may underlie the mode of activation generally referred to as pharmacomechanical coupling (see above).

Ca^{2+} entry blockers

Agents interfering with Ca^{2+} at different steps in the excitation-contraction coupling process have been useful tools to elucidate the mechanisms behind K^+ - and agonist-induced contractions in VSM. A new and exciting class of such drugs is the organic " Ca^{2+} entry blockers", exemplified by nifedipine, verapamil and diltiazem (Fleckenstein 1977). These agents are thought to reduce the stimulation-induced entry of Ca^{2+} into "excitable" cells by blocking specific membrane channels for Ca^{2+} , a property suggested to underlie their efficacies in the treatment of several cardiovascular disorders, including vasospastic angina and arterial hypertension (Fleckenstein 1977, Bolton 1979, Antman et al. 1980, Ellrodt et al. 1980, Stone et al. 1980, Nayler & Poole-Wilson 1981, Triggle & Swamy 1983, Andersson & Högestätt 1984).

Characteristically, K^+ -induced tension development generally shows high sensitivity to Ca^{2+} entry blockers in VSM, suggesting that POCs in accordance with the slow channels in cardiac muscle are effectively inhibited by these agents (Bolton 1979, Nayler & Poole-Wilson 1981, Flaim 1982, Bou et al. 1983, Cauvin et al. 1983, Triggle & Swamy 1983). However, contractions elicited by agonists vary considerably in the sensitivity to Ca^{2+} entry blockers in different VSM preparations (Bolton 1979, Flaim 1982, Bou et al. 1983, Cauvin et al. 1983, Triggle and Swamy 1983). Possible reasons for this variation have been discussed in several recent reviews (Bou et al. 1983, Cauvin et al. 1983,

Triggle & Swamy 1983). First, agonists may release intracellular Ca^{2+} to various extents, a process considered to be only moderately influenced by Ca^{2+} entry blockers (Cauvin et al. 1983). Secondly, the Ca^{2+} channels activated by agonists may differ in their sensitivity to these agents. Indeed, it has been claimed that ROCs are less affected than POCs by Ca^{2+} entry blockers (Bolton 1979, Flaim 1982, Bou et al. 1983, Triggle & Swamy 1983). Thus, the sensitivity of agonist-induced contractions to Ca^{2+} entry blockers may not only reflect the relative importance of intracellular Ca^{2+} release and Ca^{2+} influx for the contractile activation, but also the extent to which the stimulants activate POCs and ROCs, i.e. a high sensitivity to Ca^{2+} entry blockers may be associated with a relatively larger contribution of Ca^{2+} influx through POCs (activated by agonist-induced membrane depolarization) than of intracellular Ca^{2+} release and Ca^{2+} influx through ROCs (Bou et al. 1983, Cauvin et al. 1983, Triggle & Swamy 1983). However, the general validity of the assumption that ROCs are less sensitive to Ca^{2+} entry blockers than POCs has recently been questioned (Cauvin et al. 1983), because in some VSM preparations, contractions elicited by NA are more potently inhibited by Ca^{2+} entry blockers than K^{+} -induced contractions (Bevan et al. 1981, Walus et al. 1981, Cauvin et al. 1982).

Most in vitro studies concerned with the effects of Ca^{2+} entry blockers on VSM have been performed on relatively large vessels ($> 400 \mu\text{m}$), such as the rabbit and rat aorta, partly due to the lack of suitable methods to examine small blood vessels in vitro. Available techniques for recording of mechanical activity in isolated small vessels are relatively complicated and time consuming (Mulvany & Halpern 1977, Duling et al. 1981). Since conclusions based on studies on large conduit arteries may not be valid for small arteries ($< 400 \mu\text{m}$) (see, e.g., Ratz & Flaim 1982, Cauvin et al. 1984), there is a need of more information on the VSM function in the latter kind of vessel.

Adrenergic transmitter release

Most blood vessels are innervated by adrenergic (sympathetic) nerves, which can be visualized by histochemical procedures. In isolated preparations, adrenergic transmitter release can be induced by, e.g., electrical field stimulation or exposure to K^+ depolarizing medium as mentioned above. Normally, the process of transmitter release has an absolute requirement for extracellular Ca^{2+} (Baker 1972, Kirpekar 1975). The release mechanism is considered to be triggered by an increase in the cytoplasmic Ca^{2+} concentration (Baker 1972). After depolarization of the synaptic membrane, extracellular Ca^{2+} is thought to enter the nerve terminals through specific POCs (Baker 1972, Blaustein et al. 1981, Hagiwara & Byerly 1981). In contrast to depolarization-induced VSM contraction, adrenergic transmitter release has been shown to be relatively resistant to several Ca^{2+} entry blockers, suggesting that POCs in nerves differ from those in smooth muscle (Hagiwara & Byerly 1981). However, only few studies have compared the effects of these drugs on both transmitter release and contraction in the same blood vessel preparation.

Adrenoceptors

Ever since von Euler (1946) established the neurotransmitter role of NA in postganglionic sympathetic nerves, the effects of this agent on a diversity of effector organs have been intensively studied. The broad spectrum of effects generated by NA and other catecholamines were early recognized to be mediated by different receptor systems. Based on studies of the order of potency of a series of sympathomimetic amines in different tissues, Ahlquist (1948) suggested that adrenoceptors can be divided into α - and β -subtypes. In VSM, these receptors have been shown to be associated with either contraction (α) or relaxation (β) (Somlyo & Somlyo 1970, Bolton 1979). Since both α - and β -adrenoceptors may be present in the same blood vessel, the ultimate response to NA (contraction or relaxation) will be determined by the relative importance of the two types of receptor in that particular preparation.

During the last decades, it has become evident that α - and β -adrenoceptors do not represent homogenous populations of receptors with respect to their pharmacological properties, but may be further subdivided into α_1 - and α_2 - (Langer 1974) and β_1 - and β_2 - (Lands et al. 1967) adrenoceptors, respectively. Several agonists and antagonists, suggested to interact rather selectively with either α_1 - or α_2 -adrenoceptors, are now available and have been of considerable value in the classification of α -adrenoceptors (Fig 1). Although the α_2 -adrenoceptors were originally discovered on adrenergic nerves, where they were suggested to participate in a negative feed-back control of transmitter release (Langer 1974), later studies have clearly shown that these receptors also may be located in several other tissues (Langer & Shepperson 1981, Starke 1981). For example, current research has provided ample evidence to support the view that both α_1 - and α_2 -adrenoceptors occur postjunctionally in VSM (Timmermans et al. 1979, Docherty & McGrath 1980, Stevens & Moulds 1981, De Mey & Vanhoutte 1981, Skärby et al. 1983). However, the regional distribution of these receptors within the vascular system and their relative importance in blood pressure regulation have not been established.

<u>Agonist</u>		<u>Antagonist</u>
Phenylephrine	α_1	Prazosin
Noradrenaline	$\alpha_1 = \alpha_2$	Phentolamine
Clonidine	α_2	Rauwolscine

Fig 1. Suggested selectivity of some agonists and antagonists for α_1 - and α_2 -adrenoceptors (Wikberg 1979, Langer & Shepperson 1981, Starke 1981).

α -Adrenoceptors and Ca^{2+}

Much research work has recently been devoted to elucidate the cellular mechanisms delivering Ca^{2+} to the contractile proteins after α_1 - and α_2 -adrenoceptor stimulation in VSM. Several studies in vivo have shown that vasopressor responses elicited by selective α_1 -adrenoceptor agonists, but not those induced by selective α_2 -adrenoceptor agonists, are relatively resistant to Ca^{2+} entry blockers (see van Meel 1982). These observations led to the hypothesis that α_1 - and α_2 -adrenoceptors are coupled specifically to intracellular Ca^{2+} release and Ca^{2+} influx in VSM, respectively (see van Meel 1982). However, results from studies of isolated blood vessels have led to divergent conclusions (De Mey & Vanhoutte 1981, Godfraind et al. 1982, Cauvin et al. 1982, Caverio et al. 1983).

AIMS OF THE PRESENT INVESTIGATION

The present investigation was performed to obtain more information on the mechanisms linking membrane depolarization and receptor stimulation to contraction in small arteries with special reference to the involvement of different Ca^{2+} sources and Ca^{2+} entry pathways in K^{+} - and NA-induced contractions in cerebral and peripheral arteries. The specific aims of the different studies included in the present thesis were:

to develop a sensitive method for recording of mechanical activity in isolated small blood vessels (I).

to elucidate the mechanisms related to the biphasic contractile response to K^{+} depolarization in rat basilar and mesenteric arteries (II, IV).

to examine the effects of the Ca^{2+} entry blocker nifedipine on K^{+} -induced contraction and adrenergic transmitter release in isolated intra- and extracranial arteries (III).

to compare the Ca^{2+} influx pathways utilized by K^{+} and NA in isolated rat mesenteric arteries (II, IV, VI).

to pharmacologically characterize and compare the postjunctional α -adrenoceptors mediating the contractile response to NA in rat cerebral and mesenteric arteries (V).

to evaluate the relative contribution of Ca^{2+} influx and intracellular Ca^{2+} release to the contractile response induced by stimulation of different subtypes of α -adrenoceptors (VI).

to estimate differences in the excitation-contraction coupling process between cerebral and peripheral arteries (III, VI).

MATERIALS AND METHODS

Vascular preparations

Middle cerebral, basilar, mesenteric and facial arteries were obtained from adult rats (Sprague-Dawley), cats or rabbits of either sex. The excised arteries were either used immediately for experimentation or stored overnight in "standard" Krebs solution (8 - 12°C) of the following composition (in mM): NaCl 119, KCl 4.6, CaCl₂ 1.5, MgCl₂ 1.2, NaHCO₃ 15, NaH₂PO₄ 1.2 and (+)-glucose 6.

Recording of mechanical activity

Ring segments of the arteries, 1 to 3 mm long, were suspended between two stainless steel wires (50 - 200 μ m in diameter) in a temperature-controlled (37°C) 2.5 ml muscle bath (Fig. 2), containing standard Krebs solution. Each vessel segment was first threaded on one of the two steel wires under microscope. Care was taken not to stretch or otherwise damage the artery. The opposite steel wire was then gently inserted into the vessel lumen, as shown in figure 2 (inset). Mechanical activity was recorded "isometrically" by means of a Grass Instruments FT03C force-displacement transducer, connected to one of the two specimen holders. The signal from the transducer was displayed on a Grass Instruments model 7D polygraph. The other specimen holder was attached to a displacement unit, which could be manipulated by a micrometer screw for precise adjustment of the resting tension. Using the smallest steel wire (50 μ m), the compliance of the recording system was less than 10 μ m/mN. The bath solution was continuously bubbled with 95 % O₂ and 5 % CO₂, resulting in a pH of approximately 7.4. Experiments with La³⁺ were performed in Tris buffer solutions (for composition, see III), bubbled with 100 % O₂. During an equilibration period of approximately 60 min, the resting tension was adjusted to between 1 and 6 mN, depending on the size of the artery examined. The present method caused no damage to the smooth muscle or endothelial cells, as observed by light, transmission and scanning electron microscopy.

Light and electron microscopy

Arteries were fixed in 3 % glutaraldehyde, postfixed in 1 % osmium tetroxide and stained with toluidine blue for light microscopy

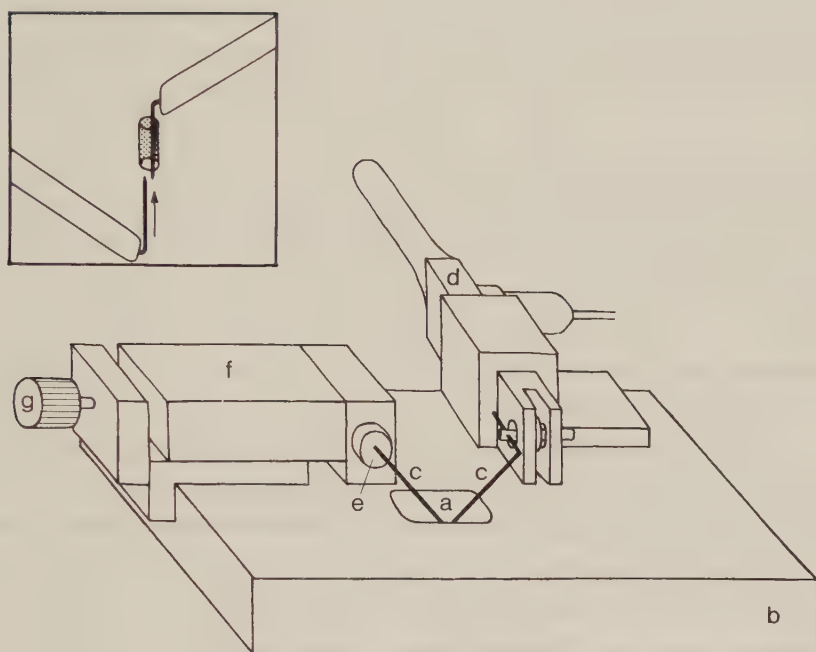


Fig 2. Schematic drawing of the myograph. Muscle bath (a), myograph block (b), specimen holders (c), force-displacement transducer (d), movable cylinder (e), displacement unit (f) and micrometer screw (g). Inset shows the small stainless steel wires at the distal ends of the specimen holders. Heated water (37°C) was circulating around the muscle bath in the myograph block.

or uranyl acetate and lead citrate for transmission electron-microscopy. The endothelial cell surface was examined by scanning electron microscopy on specimens coated with gold-palladium.

Fluorescence histochemistry

Catecholamines were visualized by the Falck-Hillarp histofluorescence method (see Björklund et al. 1972) on arteries cut open longitudinally and stretched flat on microscope slides.

Chemical denervation

In order to destroy peripheral adrenergic nerve endings, 100 mg/kg of 6-hydroxydopamine (6-OHDA) was injected into a tail vein of six rats 24 hr before sacrifice.

Measurement of ^3H -NA release

Adrenergic transmitter release was studied on arteries preincubated with ^3H -NA (2×10^{-7} M) for one hr. The ^3H -loaded preparations were transferred through a series of test tubes, containing standard Krebs solution or K^+ depolarizing medium, and the basal and K^+ -induced efflux of ^3H determined. Further details on this procedure are given in study III.

Calculations and statistical analysis

EC_{50} and IC_{50} values were determined graphically by linear interpolation. The $\log \text{EC}_{50}$ and IC_{50} values were subsequently used to calculate geometric mean values. The results shown in tables, figures and the text are expressed as mean $\pm$ SEM, with n referring to the number of arterial segments examined. pA_2 values were determined according to the method of Arunlakshana and Schild (1959). The least squares method was used for calculating linear regressions. Statistical analysis of the results was performed by means of Student's t -test. Statistical significance was accepted when $P < 0.05$.

RESULTS

Mechanical properties of the rat basilar artery (I)

Length-tension measurements were performed on relaxed and K^+ -activated rat basilar arteries in an attempt to determine the optimum resting tension for recording of contractile activity. A concentration of 124 mM K^+ was used to activate the arteries, because (1) this concentration produced an almost maximum response to K^+ (Fig 3), (2) 124 mM K^+ induced stable and highly reproducible contractions and (3) no other stimulant examined (NA, serotonin, prostaglandin $F_{2\alpha}$, angiotensin II) produced a contraction stronger than that elicited by 124 mM K^+ in the rat basilar artery. The mechanical behaviour of the vessel conformed to the sliding filament theory of contraction, originally delineated for skeletal muscle (see Huxley 1970). Thus, the active wall tension developed by the K^+ -activated arteries increased when the vessels were distended and reached a maximum of approximately 4.8 mN/mm, at an effective lumen radius of about 150 μ m, and then declined. The passive wall tension at 150 μ m was estimated to approximately 1 mN/mm, corresponding to an effective transmural pressure of approximately 50 mm Hg. The resting wall tension was therefore adjusted to approximately 1 mN/mm in all subsequent experiments on rat basilar arteries. The other arteries used in the present investigation (rat and cat middle cerebral and mesenteric arteries and rabbit basilar and facial arteries) were similarly subjected to resting wall tensions corresponding to an effective transmural pressure of about 50 mm Hg. Under isometric conditions, the K^+ -contracted rat basilar artery developed a "maximum" active wall stress of approximately 240 mN/mm².

Contractile responses to K^+ in rat basilar and mesenteric arteries (II, IV)

Concentration-response relationship for K^+ . Exposure to isotonic K^+ solutions (containing K^+ concentrations between 4.6 and 139 mM and prepared by replacing Na^+ in the standard Krebs solution by equimolar amounts of K^+) induced concentration-dependent contractions in rat basilar and mesenteric arteries (Fig 3) (Högestätt, unpublished observations). In the basilar artery, K^+

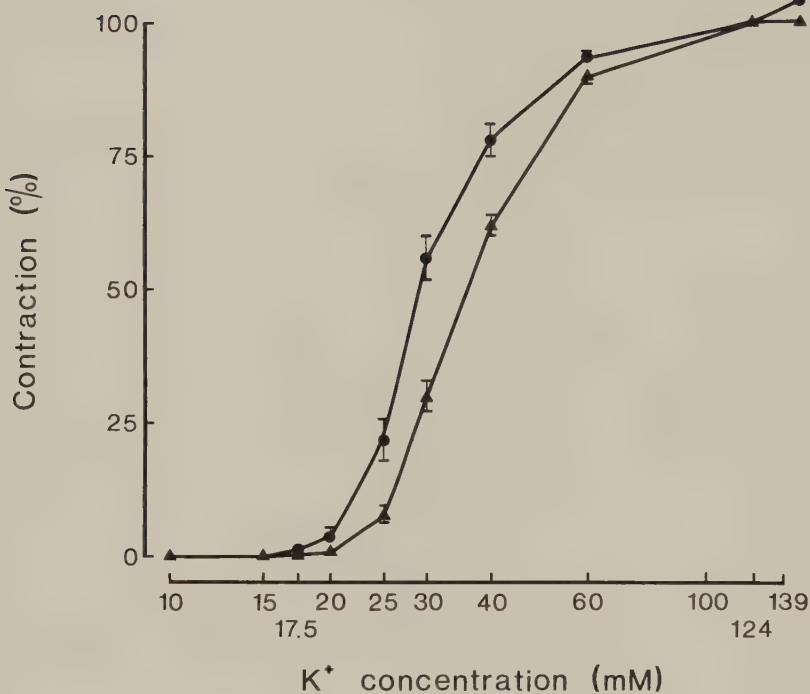


Fig 3. Concentration-response relationship for K^+ in rat basilar ($\bullet$, $n=7-14$) and mesenteric ($\blacktriangle$, $n=7-15$) arteries. The arteries were exposed to isotonic solutions with K^+ concentrations ranging between 4.6 and 139 mM. The different solutions were prepared by substituting the Na^+ in the standard Krebs solution with K^+ in equimolar amounts. The contraction induced by raising the K^+ concentration in the bath solution from 4.6 to 124 mM was set to 100 % in each experiment.

concentrations higher than 30 mM usually induced a biphasic contraction, composed of an initial rapid phase (phase A) and an ensuing slow phase (phase B), which were separated by a small transient relaxation (Fig 4). A similar biphasic response to K^+ was only occasionally observed in the mesenteric artery.

Neurogenic contribution. The contractile response to 124 mM K^+ in the basilar artery was not altered by phentolamine (10^{-6} M), prazosin (10^{-7} M), rauwolscine (10^{-6} M), propranolol (3×10^{-7} M), scopolamine (10^{-5} M) or methysergide (10^{-6} M). In the mesenteric artery, rauwolscine (10^{-6} M), propranolol (3×10^{-7} M) and scopolamine (10^{-5} M) also failed to affect the K^+ -induced contraction, whereas phentolamine (10^{-6} M) and prazosin (10^{-7} M) reduced the maximum response by $27 \pm 3\%$ ($n=4$) and $25 \pm 1\%$ ($n=11$), respectively (Högestätt, unpublished observations). Notably, in the presence of phentolamine or prazosin, the mesenteric artery responded to K^+ with a biphasic contraction, similar to that observed in the basilar artery (Fig 4). This characteristic biphasic course of the K^+ -induced contraction was also observed in mesenteric arteries obtained from 6-OHDA treated rats (Fig 4).

Effects of Ca^{2+} removal, nifedipine and La^{3+} . Exposure to Ca^{2+} -free solution (containing 10^{-5} M EGTA) consistently reduced the contractile response to 124 mM K^+ in basilar and mesenteric arteries. A 20-min preincubation was usually sufficient to abolish the K^+ -induced contraction. Prolonged treatment (> 4 hr) of the basilar artery in Ca^{2+} -free medium (containing 1 mM EGTA) reduced neither the amplitude of phase A nor that of phase B of the contraction induced by the simultaneous application of K^+ and Ca^{2+} . Re-addition of 1.5 mM Ca^{2+} to " Ca^{2+} -depleted" basilar and mesenteric (exposed to 10^{-7} M prazosin) arteries depolarized by K^+ also induced a biphasic contraction.

Nifedipine effectively inhibited both phases of the contractile response to K^+ in basilar and mesenteric arteries. However, phase B of the K^+ -induced contraction was approximately ten times more sensitive to the drug than phase A in both types of artery (Fig 5). Moreover, the sensitivity of the two contraction

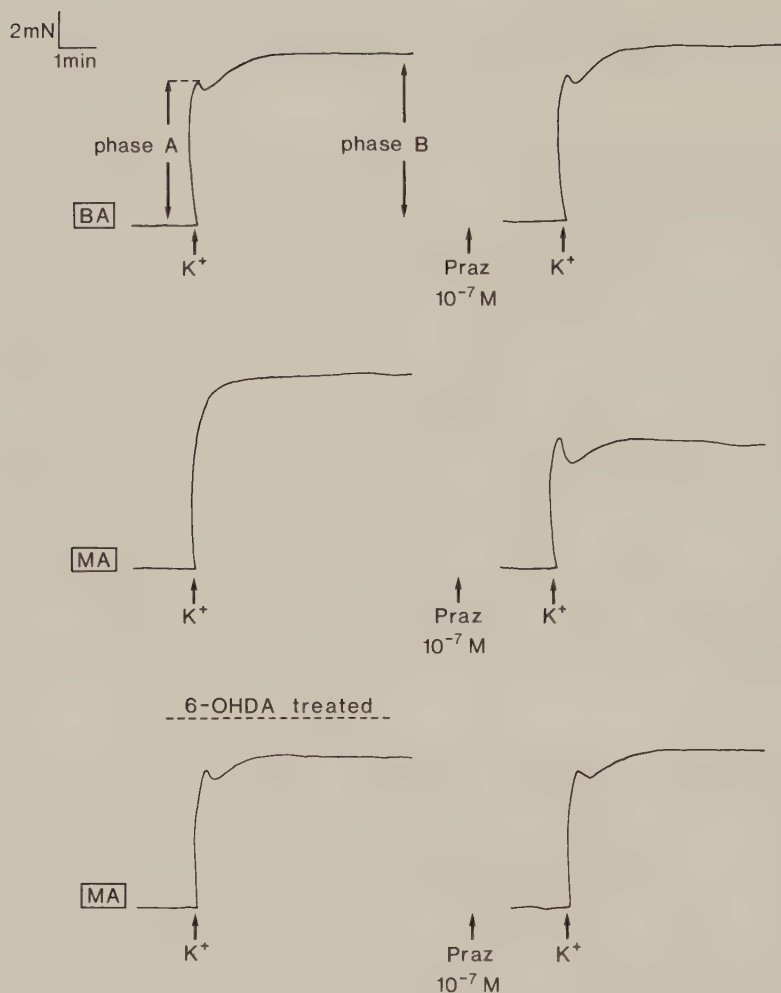


Fig 4. Contractile response to 124 mM K⁺ before and after application of 10⁻⁷ M prazosin (Praz) in rat basilar (BA) and mesenteric (MA) arteries. The lower tension tracings were obtained from a mesenteric artery, removed from a 6-hydroxydopamine (6-OHDA) treated rat.

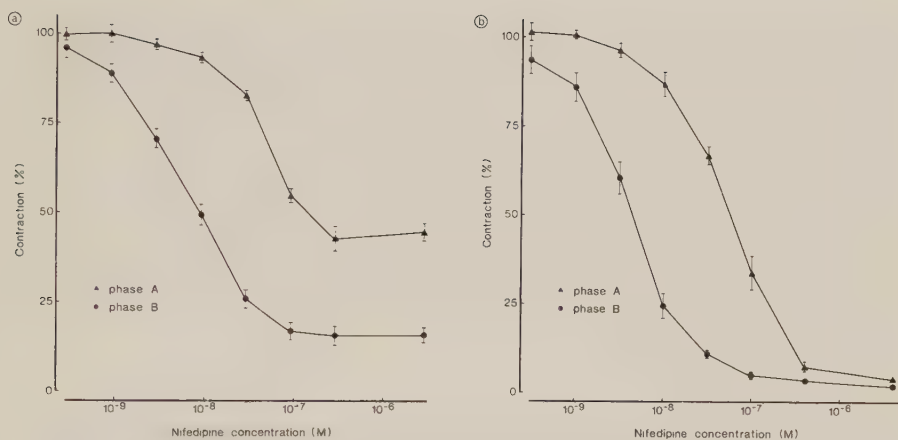


Fig 5. Inhibition by nifedipine of the initial rapid phase (phase A, ▲) and the ensuing slow phase (phase B, ●) of the contractile response to 124 mM K^+ in rat basilar (a; $n=6-8$) and mesenteric (b; $n=5-9$) arteries. The amplitude of the K^+ -induced contraction at the peak of the initial transient response and 5 min after introduction of K^+ was used as measures of phase A and phase B, respectively. Responses are expressed as per cent of the corresponding contraction phase before application of nifedipine. The time of exposure to each nifedipine concentration was approximately 15 min. Prazosin (10^{-7} M) was present in all experiments on mesenteric arteries.

components to nifedipine did not differ markedly between the two types of artery, although the maximum inhibition produced by the drug was significantly larger in mesenteric than in basilar arteries (Högestätt, unpublished observations). Unlike nifedipine, La^{3+} was unable to differentiate between the two contraction components in rat basilar and mesenteric arteries, and at a concentration of 1 mM of La^{3+} the response to K^+ was almost abolished.

Substitution of NaCl with sucrose, LiCl or choline Cl. In additional experiments (Högestätt, unpublished observations), replacement of all NaCl (119 mM) in the standard Krebs solution with sucrose (238 mM) or LiCl (119 mM) did not influence the wall tension of basilar and mesenteric arteries. However, substitution of NaCl (119 mM) with choline Cl (119 mM) contracted the basilar artery, but had no effect on the mesenteric artery, and the amplitude of the response was comparable with that induced by 124 mM K^+ (Fig 6).

Effects of nifedipine on K^+ -induced contraction and adrenergic transmitter release in rabbit basilar and facial arteries (III)

K^+ -induced contraction. Nifedipine effectively inhibited the contractile response to 124 mM K^+ in rabbit basilar and facial arteries. However, the inhibition produced by nifedipine was slightly larger in basilar than in facial arteries, as evidenced by a lower IC_{50} value (10 nM versus 30 nM) and a larger maximum inhibition (80 % versus 60 %). Phentolamine (10^{-6} M) or prazosin (10^{-6} M) reduced the K^+ -induced contraction by approximately 10 % in the facial artery, but had no effect on the response to K^+ in the basilar artery. This raised the question as to whether the differential inhibition produced by nifedipine might have been caused by differences in the contribution of endogenous NA to the K^+ -induced contraction in the two types of artery. To evaluate this possibility, the inhibition produced by nifedipine was re-examined in the presence of 10^{-6} M phentolamine or 10^{-6} prazosin. It was found that in the presence of the α -adrenoceptor blockers, the K^+ -induced contractions were reduced to approximately the same extent by 0.3 and 3×10^{-7} M nifedipine in the two types of artery.

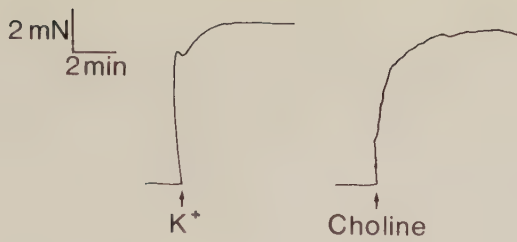


Fig 6. Contractions induced by replacement of all NaCl (119 mM) in the bath solution with 119 mM KCl (K^+) or 119 mM choline Cl (Choline) in a rat basilar artery.

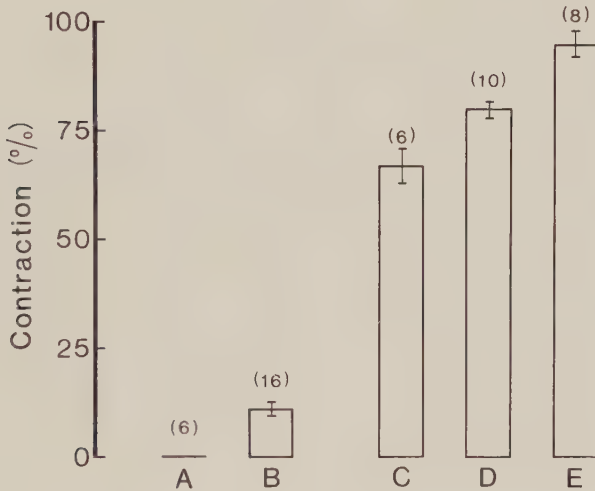


Fig 7. Effects of 3×10^{-7} M nifedipine on contractions in rat mesenteric arteries induced by (A) 1.5 mM Ca^{2+} in "Ca $^{2+}$ -depleted" arteries depolarized by 124 mM K^+ (10^{-5} M EGTA), (B) 124 mM K^+ in Ca $^{2+}$ containing medium, (C) 1.5 mM Ca^{2+} in Ca $^{2+}$ -depleted arteries exposed to 10^{-5} M NA (10^{-5} M EGTA), (D) 10^{-5} M NA in standard Krebs solution and (E) 10^{-4} M NA after 5 min in Ca $^{2+}$ -free medium (10^{-3} M EGTA). Prazosin (10^{-7} M) was present in A and B. The amplitude of the different contractions before application of nifedipine was set to 100 %. The time of exposure to nifedipine was approximately 15 min. Figures within brackets indicate the number of arteries examined.

K⁺-induced ³H-NA release. Fluorescence histochemical demonstration of catecholamines revealed a dense network of adrenergic-like nerve fibres in the walls of basilar and facial arteries. The vessels were able to accumulate ³H-NA and release it upon depolarization by 40 and 124 mM K⁺ (Table 1). Desipramine (10⁻⁵ M) attenuated the uptake of ³H-NA by more than 80 % in both types of vessel (Table 1). Nifedipine failed to significantly alter the basal and K⁺-induced efflux of ³H from basilar and facial arteries in concentrations sufficiently high (0.3 and 3 x 10⁻⁷ M) to produce a maximum inhibition of K⁺-induced contractions.

Comparison between K⁺- and NA-induced contractions in rat mesenteric arteries (IV, VI)

Effects of Ca²⁺ removal, nifedipine and La³⁺. Based on the rate of tension increase, the contractile response to 10⁻⁵ and 10⁻⁴ M NA in rat mesenteric arteries could usually be divided into an initial fast and an ensuing slow (tonic) component (cf. Bohr 1963, Watkins & Davidson 1980). Ca²⁺ deprivation reduced the tonic component of the NA-induced contraction to a larger extent than the initial fast component. After 5 min in Ca²⁺-free medium (containing 1 mM EGTA) 10⁻⁴ M NA induced only a transient contraction, amounting to 47 ± 2 % (n=13) of the response to the agonist in standard Krebs solution. However, the same treatment almost abolished the contractile response to 124 mM K⁺ (Högestätt, unpublished observations).

Nifedipine inhibited the K⁺-induced contraction (in the presence of 10⁻⁷ M prazosin) considerably more than the contractile response to 10⁻⁵ M NA (Fig 7, B and D). Moreover, nifedipine failed to influence the transient contraction elicited by 10⁻⁴ M NA in Ca²⁺-free medium (Fig 7, E) (Högestätt, unpublished observation). In contrast to nifedipine, La³⁺ inhibited the contractile responses to K⁺ and 10⁻⁵ M NA to approximately the same extent. The EC₅₀ value for La³⁺ was approximately 3 x 10⁻⁶ M for both K⁺- and NA-induced contractions.

Table 1. Uptake and release of ^3H -NA in control and desipramine (10^{-5} M)-treated rabbit basilar and facial arteries. For further explanations, see study III.

	Uptake; (cpm/mg* x 10^{-5})		Basal fractional efflux of ^3H ; (%)		Fractional efflux of ^3H induced by 124 mM K^+ ; (%)	
	Basilar artery	Facial artery	Basilar artery	Facial artery	Basilar artery	Facial artery
Controls	1.6 $\pm$ 0.3 n = 7	1.7 $\pm$ 0.2 n = 12	0.80 $\pm$ 0.03 n = 18	0.43 $\pm$ 0.03 n = 15	6.6 $\pm$ 0.6 n = 18	4.0 $\pm$ 0.3 n = 15
Desipramine-treated	0.25 $\pm$ 0.07 n = 4	0.29 $\pm$ 0.07 n = 5	0.90 $\pm$ 0.19 n = 4	0.77 $\pm$ 0.06 n = 4	0.07 $\pm$ 0.04 n = 4	0.16 $\pm$ 0.06 n = 4

* Wet weight

Contractile response to NA in K^+ -depolarized muscle. Application of 10^{-5} M NA to K^+ (124 mM)-contracted mesenteric arteries, removed from 6-OHDA treated rats, further increased the active tension by approximately 70 %. In arteries preincubated with 3×10^{-7} M nifedipine in order to reduce the K^+ -induced contraction to a minimum, NA evoked a sustained tonic contraction, which was only about 20 % smaller than that elicited by NA in standard Krebs solution.

Contractile responses to Ca^{2+} in K^+ -depolarized and NA-exposed arteries. Ca^{2+} induced concentration-dependent contractions in Ca^{2+} -depleted arteries exposed to 10^{-5} M NA or 124 mM K^+ (in the presence of 10^{-7} M prazosin). The EC_{50} value for Ca^{2+} was approximately three times lower in K^+ -depolarized than in NA-exposed arteries. The graded responses to Ca^{2+} in NA-exposed vessels were not significantly enhanced by increasing the NA concentration by a factor of ten, indicating that 10^{-5} M was sufficient to produce a maximum increase in the Ca^{2+} permeability. The EC_{50} value for Ca^{2+} was considerably lower after application of both K^+ and NA than in presence of either stimulant separately. The maximum response to Ca^{2+} was 37 % smaller in K^+ -depolarized arteries (in the presence of 10^{-7} M prazosin) than in preparations exposed to both K^+ and NA. The Ca^{2+} -induced contractions were considerably more inhibited by nifedipine in K^+ -depolarized (in the presence of 10^{-7} M prazosin) than in NA-exposed arteries (Fig 7, A and C).

Pharmacological classification of the α -adrenoceptors mediating the contractile response to NA in rat cerebral and mesenteric arteries (V)

Concentration-response relationship for NA. NA induced concentration-dependent contractions in rat middle cerebral and mesenteric arteries. The EC_{50} value for the agonist was approximately 2.5×10^{-6} M in middle cerebral and 3.9×10^{-6} M in mesenteric arteries. However, NA usually failed to elicit contraction in the basilar artery even in the presence of propranolol (3×10^{-7} M) and cocaine (10^{-6} M). When expressed as

per cent of the contractile response to 124 mM K^+ , the maximum contraction elicited by NA was considerably smaller in middle cerebral (31 %) than in mesenteric (111 %) arteries, although the maximum contractile response to serotonin did not differ significantly between the two types of artery.

Effects of propranolol and cocaine. In the middle cerebral artery, propranolol ($3 \times 10^{-7} \text{ M}$) increased the maximum contractile response to NA by approximately 100 % without affecting the EC_{50} value for the agonist. The concentration-response relationship for NA in the mesenteric artery was not affected by propranolol. Cocaine (10^{-5} M), on the other hand, significantly increased the sensitivity of the mesenteric artery to NA by a factor of three, whereas the drug (10^{-6} M) slightly reduced the maximum contractile response to the agonist in the middle cerebral artery.

Effects of α -adrenoceptor agonists. The order of potency of a series of α -adrenoceptor agonists was (-)-adrenaline > oxymetazoline > (-)-NA > ($\pm$)-NA $\approx$ (-)-phenylephrine > methoxamine >> (+)-NA > clonidine in the middle cerebral artery and ($\pm$)-NA > (-)-phenylephrine >> clonidine in the mesenteric artery. Oxymetazoline behaved as a partial agonist in the middle cerebral artery, since the maximum contractile response to the agonist was approximately 40 % smaller than that to ($\pm$)-NA. Interestingly, oxymetazoline also contracted the rat basilar artery, although the other α -adrenoceptor agonists failed to elicit contraction in this preparation.

Effects of α -adrenoceptor antagonists. Prazosin inhibited the contractile response to NA in an apparently competitive manner in middle cerebral and mesenteric arteries. The pA_2 value was estimated to 9.3 in middle cerebral and 9.7 in mesenteric arteries (Table 1). Rauwolscine was between three and four orders of magnitude less potent than prazosin in both types of artery.

Table 2. Results from the Schild plots for the antagonism of noradrenaline-induced contraction by prazosin and rauwolscine in rat middle cerebral and mesenteric arteries. The results are given as mean values with 95 % confidence interval within brackets. z represents the number of points used for calculating the regression line. For experimental conditions, see study V.

Artery	Antagonist	z	Correlation Coefficient	Slope of regression line	pA ₂ value
Middle cerebral artery	Prazosin	43	0.95	1.01 (0.90 - 1.12)	9.32 (9.17 - 9.49)
Middle cerebral artery	Rauwolscine	13	0.81	1.43 (0.74 - 2.11)	5.40 (5.03 - 5.63)
Mesenteric artery	Prazosin	40	0.97	0.93 (0.86 - 1.01)	9.70 (9.58 - 9.84)
Mesenteric artery	Rauwolscine	13	0.85	0.64 (0.38 - 0.89)	6.80 (6.47 - 7.39)

Influence of extracellular Ca^{2+} and nifedipine on NA-induced contractions in rat and cat middle cerebral and mesenteric arteries (VI)

Effect of Ca^{2+} removal. NA (10^{-5} M) usually induced an initial rapid tension increase followed by a tonic response in rat middle cerebral and rat and cat mesenteric arteries. However, in the cat middle cerebral artery, the NA-induced contractions developed considerably slower than in the other types of vessel. Incubation of the cat middle cerebral artery in Ca^{2+} -free medium (containing 10^{-5} M EGTA) for 10 min virtually abolished the contractile response to NA, whereas in the other types of artery, NA induced a significant contraction after the same treatment. The amplitude of this contraction, relative to the control response to NA in standard Krebs solution, decreased in the following order between the vessels: cat mesenteric artery (35 %) $\approx$ rat mesenteric artery (33 %) > rat middle cerebral artery (15 %) >> cat middle cerebral artery (1 %). Excluding the cat middle cerebral artery, in which the contractile responses to 124 mM K^{+} and 10^{-5} M NA were equally reduced in Ca^{2+} -free medium, the K^{+} -induced contraction was significantly more suppressed by Ca^{2+} deprivation than the NA-induced contraction.

Effect of nifedipine. Nifedipine effectively inhibited the contractile response to NA in rat and cat middle cerebral arteries, whereas it was a comparatively weak inhibitor of NA-induced contractions in rat and cat mesenteric arteries. The vessels could be ranked in the following order with respect to the sensitivity to nifedipine: cat middle cerebral artery >> rat middle cerebral artery >> cat mesenteric artery $\approx$ rat mesenteric artery. As demonstrated by regression analysis, a close correlation ($r=0.99$, $P < 0.01$) was found between the maximum inhibition of the tonic component produced by nifedipine and the amplitude of the contraction elicited by NA after 10 min in Ca^{2+} -free medium.

DISCUSSION

Methodological considerations

A multitude of techniques have been utilized for studying the mechanical activity in isolated VSM preparations (see Moulds 1983). However, most of these are confined to relatively large vessels with diameters greater than 400 μm . The in vitro method used in the present investigation allows studies of mechanical activity in ring segments of blood vessels with calibres down to 100 μm . The method is relatively easy to apply and offers certain advantages over helically cut preparations, one being that the anatomical structure of the vessels is reasonably retained (Ohhashi & Azuma 1980). The handling of the vessels does not cause any observable damage to the smooth muscle or endothelial cells, not even in the area of the vessel wall which was wrapped around the steel wires, as demonstrated by light, transmission and scanning electron microscopy. This is important since several recent studies have indicated the necessity of an intact endothelial cell layer for a proper function of arteries in vitro (see, e.g., Furchgott & Zawadzki 1980). Furthermore, the contractile response to K^+ was highly reproducible and the "maximum" active wall stress developed by the K^+ -activated rat basilar artery was comparable with that found in other smooth muscle preparations (see Murphy 1976, Dubrin 1978), suggesting that the VSM cells were also maintained functionally intact.

Mechanisms behind the biphasic contractile response to K^+

The present study (II) clearly showed that replacement of all NaCl by KCl in the bath solution induced a biphasic contraction in the rat basilar artery. A biphasic response to K^+ was generally observed only after application of K^+ concentrations higher than 30 mM. The K^+ -induced contraction was not influenced by classical adrenoceptor, cholinceptor or serotonin receptor blocking agents, indicating that adrenergic, cholinergic or serotonin-related mechanisms are probably not involved in the biphasic response to K^+ . However, in the rat mesenteric artery, phentolamine and prazosin, but not rauwolscine, propranolol and scopolamine, significantly reduced the contractile response to K^+ by approximately 25 %. This suggests that endogenous NA released by

K^+ significantly contributed to the K^+ -induced contraction in the rat mesenteric artery, presumably by stimulation of postjunctional α_1 -adrenoceptors. Notably, when the adrenergic component was reduced either by exposing the arteries to α -adrenoceptor blockers or by using chemically denervated vessels, the contractile response to K^+ became clearly biphasic, resembling that induced by K^+ in the rat basilar artery.

It has been demonstrated that the initial rapid phase of the K^+ -induced contraction in the guinea-pig taenia coli and rat ileal smooth muscle is accompanied by membrane depolarization and the appearance of spike discharges (Shimo & Holland 1966, Imai & Takeda 1967, Syson & Huddart 1973). Electrophysiological studies on rat cerebral and mesenteric arteries have shown that exposure to solutions with K^+ concentrations of approximately 120 mM reduced the membrane potential to between -10 and -5 mV (Mulvany et al. 1982, Harder et al. 1983). Nonetheless, re-application of Ca^{2+} to rat basilar and mesenteric arteries previously depolarized by K^+ in Ca^{2+} -free medium induced a biphasic contraction. This does not support the view that the initial rapid phase of the contractile response to K^+ was initiated by a burst of spikes. Indeed, Mulvany et al. (1982) reported that although K^+ induced a biphasic contraction in rat mesenteric arteries, the accompanying membrane depolarization increased monophasically without the appearance of spike potentials, also supporting this opinion.

Release of Ca^{2+} from intracellular stores did not appear to be involved in the K^+ -induced contraction in rat basilar and mesenteric arteries, since Ca^{2+} deprivation rapidly reversed and La^{3+} effectively inhibited both components of the biphasic response to K^+ . In addition, prolonged treatment in Ca^{2+} -free medium reduced neither phase A nor phase B of the contraction induced by the simultaneous addition of K^+ and Ca^{2+} to the Ca^{2+} -depleted rat basilar artery, also supporting this view. A similar conclusion was reached by Sunano and Miyazaki (1968), Hurwitz et al. (1980) and Shimodan and Sunano (1981) regarding the intra- and extracellular Ca^{2+} dependence of the biphasic contractile response to K^+ in guinea-pig vas deferens and ileal smooth muscle.

It has been argued that the intracellular Ca^{2+} concentration in VSM is partly regulated by an electrogenic $\text{Na}^{+}\text{-Ca}^{2+}$ exchange mechanism with a presumed stoichiometry of three Na^{+} for each Ca^{2+} (Blaustein 1977). Such an exchange process would be sensitive both to the membrane potential and to the Na^{+} gradient over the cell membrane. Hence, the depolarizing effect and the low Na^{+} content of the isotonic K^{+} -rich solutions used in the present study may theoretically cause contraction by suppressing or reversing a hypothetical Na^{+} -dependent, Ca^{2+} extrusion mechanism. However, the observation that replacement of all NaCl in the bath solution with either sucrose, choline Cl or LiCl did not induce contraction in the rat mesenteric artery does not favour the involvement of such a mechanism in the K^{+} -induced contraction in this preparation. The experiments on the rat basilar artery are less conclusive, since substitution of NaCl with choline Cl, but not with sucrose or LiCl, induced a slow (tonic) contraction, comparable in size with that elicited by K^{+} .

Before discussing the effects of nifedipine on the K^{+} -induced contractions, it seems justified to briefly review the mechanisms of action of this agent. Most of the information regarding the membrane effects of nifedipine has been obtained from electrophysiological studies on the heart. In mammalian ventricular myocardium, nifedipine effectively reduced the slow inward current of Ca^{2+} (Kohlhardt & Fleckenstein 1977, Bayer & Ehara 1978). Nifedipine was suggested to inhibit rather selectively the slow Ca^{2+} channels without affecting the "fast Na^{+} channels", since the drug did not influence the upstroke velocity of the action potential in the myocardial cell (Rodenkirchen et al. 1977). Voltage clamp studies revealed that nifedipine did not alter the rates of activation and inactivation of the slow channels, but rather reduced the number of channels available for Ca^{2+} transport (Kohlhardt & Fleckenstein 1977, Bayer & Ehara 1978).

Analogous with its mechanism of action on heart muscle, the relaxing effects of nifedipine on VSM have been attributed to inhibition of stimulation-induced Ca^{2+} entry (Fleckenstein 1977, Bolton 1979, Cauvin et al. 1983, Andersson & Högestätt 1984). Godfraind (1983) reported that the concentration-response relationships for the inhibitory effects of nifedipine on K^{+} -

induced contraction and $^{45}\text{Ca}^{2+}$ influx were almost identical in the rat aorta and superior mesenteric artery, suggesting that the drug attenuated contraction by blocking Ca^{2+} translocation over the cell membrane. Resting Ca^{2+} uptake (passive leak) in smooth muscle does not appear to be appreciably influenced by nifedipine (Rosenberger et al. 1979, Church & Zsotér 1980, Godfraind 1983).

^3H -Nifedipine has been reported to bind with high affinity to cardiac membranes (Holck et al. 1982) and the unlabelled drug potentially inhibited the binding of the tritiated nifedipine analogues nitrendipine and nimodipine to cardiac, smooth muscle and brain membranes (Belleman et al. 1982, Ehlert et al. 1982, DePower et al. 1982, Glossmann et al. 1982, Williams & Tremble 1982). In contrast to verapamil and D 600, nifedipine in reasonable concentrations ($< 10^{-6}$ M) has not been shown to interact with α -adrenoceptors (van Meel et al. 1981, Larsson et al. 1984).

Nifedipine is a lipophilic drug and may therefore be expected to enter the smooth muscle cell. Intracellular sites of action of this compound have been suggested by some authors (Mikkelsen et al. 1979, Church & Zsotér 1980, Walus et al. 1981), whereas others have failed to demonstrate intracellular effects of nifedipine (Pang & Sperelakis 1982, Godfraind 1983, Heaslip & Rahwan 1983, Kreye et al. 1983, Spedding 1983). As shown in the present investigation, the transient contraction induced by NA in the rat mesenteric artery pretreated in Ca^{2+} -free medium was not inhibited by nifedipine, suggesting that the drug did not interfere with the process of intracellular Ca^{2+} release. Put together, Ca^{2+} entry blockade still seems to be the main mechanism behind the relaxing effect of nifedipine on VSM, even if other effects may contribute.

Unlike La^{3+} , which failed to differentiate between the two components of the K^{+} -induced contraction in rat basilar and mesenteric arteries, nifedipine produced a larger inhibition of the slow phase than of the initial rapid phase of the biphasic response to K^{+} . One possible explanation of the differential effect of nifedipine on the two contraction components might be that K^{+} depolarization activates two different types of POC, one permitting a rapid and transient

movement of Ca^{2+} across the cell membrane (the "rapidly activated Ca^{2+} channel"), corresponding to the fast phase of contraction, the other allowing Ca^{2+} to pass at a slower rate (the "slowly activated Ca^{2+} channel"), corresponding to the slow phase of contraction. In a recent study on the longitudinal muscle of the guinea-pig ileum, Hurwitz et al. (1980) also interpreted the biphasic contractile response to K^+ as reflecting the operation of two separate groups of K^+ -activated Ca^{2+} channel, differing in both the rates of activation and inactivation. The possible physiological significance of multiple POC types is unclear. It may be speculated that the rapidly activated Ca^{2+} channels are involved in fast electrical events, such as action potential discharges, whereas the slowly activated Ca^{2+} channels are opened by graded, sustained membrane depolarization. However, more direct evidence is required to settle the question of a possible heterogeneity of POCs in VSM. Interestingly, studies of $^{45}\text{Ca}^{2+}$ influx and transmitter release induced by K^+ depolarization in isolated brain synaptosomes, using a rapid filtration method, have suggested the existence of two classes of POC with different kinetic properties in the mammalian central nervous system (Blaustein et al. 1981).

Differential effects of nifedipine on K^+ -induced contraction and adrenergic transmitter release

As demonstrated in the present study (III), nifedipine produced a larger inhibition of K^+ -induced contractions in rabbit basilar arteries than in rabbit facial arteries. However, the difference in maximum inhibition was abolished when the experiments were performed in the presence of phentolamine or prazosin. This suggests that different contributions of endogenous NA to the K^+ -induced contraction in the two types of artery were mainly responsible for the difference in inhibition, rather than dissimilarities in the properties of the POCs activated by K^+ in the two types of vessel. These results emphasize the importance of eliminating the adrenergic component when the sensitivities of K^+ -induced contractions to Ca^{2+} entry blockers in different VSM preparations are compared.

Although nifedipine was a potent inhibitor of K^+ -induced tension development, it failed to influence the efflux of 3H induced by K^+ depolarization in rabbit basilar and facial arteries preincubated with 3H -NA. A similar lack of effect of nifedipine on adrenergic transmitter release has also been demonstrated in the electrically stimulated rabbit urethra (Larsson et al. 1984). Starke and Schümann (1973) reported that the overflow of NA induced by stimulation of the sympathetic cardiac nerves in the rabbit was reduced only at very high nifedipine concentrations, which almost abolished the mechanical activity of the myocardium. The selective action of nifedipine on K^+ -induced contraction over adrenergic transmitter release, as observed in the present study (III), may indicate that POCs present on peripheral adrenergic nerve endings have properties different from those in VSM. Upon reviewing the effects of organic Ca^{2+} entry blockers on Ca^{2+} currents in various preparations and the relative efficacies of polyvalent cations to block these currents, Hagiwara and Byerly (1981) suggested that there are fundamental differences between Ca^{2+} channels in different tissues. In this respect, the Ca^{2+} channels differ from the Na^+ channels, which are generally considered to be a relatively unique structure with properties varying only slightly from preparation to preparation (Hagiwara & Byerly 1981). In this connection it is interesting to note that although nifedipine failed to influence the K^+ -induced $^{45}Ca^{2+}$ uptake into rat brain synaptosomes (Nachshen & Blaustein 1979) and catecholamine release from a vesicular preparation of the guinea-pig brain (Ebstein & Daly 1982), it potently inhibited the binding of 3H -nitrendipine or 3H -nimodipine to rat and guinea-pig brain membranes (Belleman et al. 1982, Ehlert et al. 1982, Marangos et al. 1982, Murphy & Snyder 1982). A similar apparent lack of correlation between binding and functional studies with respect to the action of nifedipine has not been observed for cardiac and smooth muscle (Bolger et al. 1982, Holck et al. 1982). These findings may be interpreted in terms of Ca^{2+} channel heterogeneity, although there may be other explanations (see Triggle 1984).

Different Ca^{2+} entry pathways utilized by K^+ and NA

Although the tonic component of the contractile responses to both K^+ and NA in rat mesenteric arteries was critically dependent on the presence of Ca^{2+} in the bath solution, and thus appears to rely almost entirely on influx of extracellular Ca^{2+} , nifedipine preferentially reduced the tonic component of the K^+ -induced contraction. In this respect nifedipine differed from La^{3+} , which inhibited K^+ - and NA-induced contractions to approximately the same extent. These results suggest that K^+ and NA utilize partly different Ca^{2+} entry pathways to increase the myoplasmic Ca^{2+} concentration in the rat mesenteric artery. This interpretation was further supported by experiments showing that (1) nifedipine was a more effective inhibitor of Ca^{2+} -induced contractions in K^+ - than in NA-exposed arteries and (2) preparations depolarized by K^+ in the presence of nifedipine responded to NA with a sustained tonic contraction (also dependent on extracellular Ca^{2+}), which was only slightly smaller than that elicited by NA under control conditions, implicating pharmacomechanical coupling.

Three pieces of evidence suggest that the effects of K^+ and NA on the Ca^{2+} permeability of the muscle membrane were additive. First, the EC_{50} value for Ca^{2+} was considerably lower after application of both K^+ and NA than in presence of either stimulant separately. Secondly, the maximum contractile response to Ca^{2+} was approximately 40 % smaller in arteries depolarized by K^+ in the presence of prazosin than in those exposed to both K^+ and NA. Thirdly, NA further increased the active tension in K^+ -contracted vessels. The presence of additivity indicates that the Ca^{2+} entry pathways utilized by NA and K^+ (after elimination of the adrenergic contribution) are at least partly independent. It also appears to rule out the possibility of a single pathway regulated by two separate mechanisms (cf. Meisheri et al. 1981).

Based on unidirectional $^{45}\text{Ca}^{2+}$ flux measurements in the rabbit aorta, Meisheri et al. (1981) provided direct evidence of the existence of two separate Ca^{2+} entry pathways in VSM, one activated by K^+ depolarization and sensitive to the Ca^{2+} entry blocker D 600, and the other activated by NA and relatively resistant to D 600. This indicates that depolarization-induced

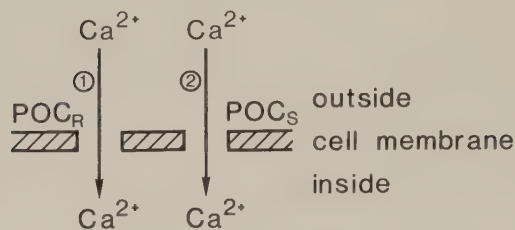
Ca^{2+} influx can differ from receptor-activated influx in VSM. In a later publication, the authors suggested that these pathways are equivalent to POCs and ROCs, respectively (Cauvin et al. 1983). The results of the present study (IV) are also compatible with the view that NA and K^{+} (after elimination of the adrenergic component) stimulated Ca^{2+} influx in rat mesenteric arteries by activating different populations of Ca^{2+} channel (ROCs and POCs), differing in the sensitivity to nifedipine (Fig 8, pathway 1, 2 and 3). However, there are at least two alternative models, which also may account for the nifedipine resistance of the Ca^{2+} entry pathway utilized by NA in this preparation. Both of these are based on an inability of nifedipine to influence the process of intracellular Ca^{2+} release and the subsequent refilling of the NA sensitive intracellular Ca^{2+} store either through a direct pathway between the store and the extracellular medium (Fig 8, pathway 4) (cf. Casteels & Droogmans 1981) or by an active uptake of Ca^{2+} entering the myoplasm by passive leak (Fig 8, pathway 6) (cf. Cauvin et al. 1983).

Postjunctional α -adrenoceptors in rat cerebral and mesenteric arteries

A large bulk of information from several animal species and man supports the view that NA is a considerably weaker contractile agent in cerebral than in peripheral arteries (Toda & Fujita 1973, Müller-Schweinitzer & Weidmann 1977, Rose & Moulds 1979, McCalden & Bevan 1981, Skärby et al 1983). As demonstrated in the present study (V), this seems to apply also to the rat.

As outlined in the "Introduction" the ultimate response of a preparation to an agonist, which can be expected to interact with more than one type of receptor, will be the net result of the effects mediated by the different receptor systems. In the rat middle cerebral artery, propranolol increased the maximum contractile response to NA by approximately 100 %, but had no effect on the concentration-response relationship for NA in the rat mesenteric artery. This suggests that stimulation of β -adrenoceptors functionally opposed the α -adrenoceptor mediated contractile effect of NA in the middle cerebral artery, but not in the mesenteric artery. However, even in the presence of

(a)

Nifedipine sensitive

(b)

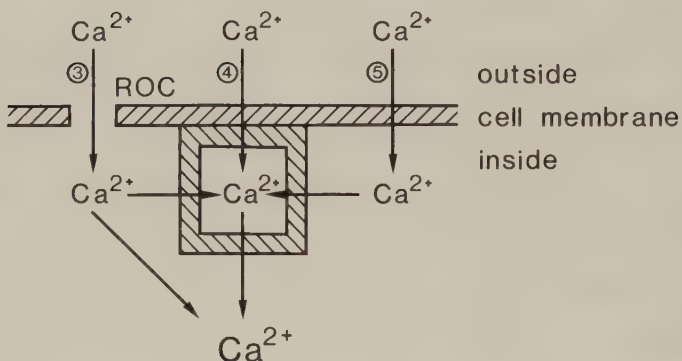
Nifedipine insensitive

Fig 8. (a) Possible Ca^{2+} entry pathways activated by K^+ depolarization and sensitive to nifedipine: (1) Ca^{2+} influx via a rapidly activated, potential-operated channel (POC_R), responsible for the fast component of the contractile response to K^+ . (2) Ca^{2+} influx via a slowly activated, potential-operated channel (POC_S), responsible for the slow component of the K^+ -induced contraction. (b) Possible Ca^{2+} entry pathways utilized by noradrenaline (NA) and relatively resistant to nifedipine: (3) Ca^{2+} influx via a receptor-operated channel (ROC). (4) Refilling of the NA sensitive intracellular Ca^{2+} store via a direct pathway into the store from the extracellular medium and subsequent release of Ca^{2+} from the store into the myoplasm. (5) "Passive leak" of Ca^{2+} across the sarcolemma. Immediately upon entering the myoplasm, this Ca^{2+} is actively pumped into the NA sensitive intracellular Ca^{2+} store, thus participating in the refilling of the store, from where it is subsequently released to activate the contractile proteins.

propranolol, the $E_{\max}$ for NA (relative to the maximum contraction induced by K^+ or 5 HT) was approximately 60 % smaller in the middle cerebral artery than in the mesenteric artery. The low responsiveness of the middle cerebral artery to NA cannot be attributed to removal of NA from the biophase by an avid neuronal uptake mechanism, since cocaine rather reduced the maximum contraction induced by NA. The failure of cocaine to potentiate the contractile response to NA seems to be a general feature of cerebrovascular smooth muscle, as similar results have been reported for rabbit, cat and dog cerebral arteries (Duckles & Bevan 1976, Toda et al. 1978, Araki et al. 1982). By contrast, in rat mesenteric arteries, cocaine increased the sensitivity to NA by a factor of three. Thus, although both rat middle cerebral and mesenteric arteries receive an abundant supply of adrenergic nerves (Furness 1973, Kobayashi et al. 1981), they were quite differently affected by cocaine. This suggests differences between the two types of artery in the function of the adrenergic neuroeffector apparatus, possibly related to a larger neuromuscular distance in cerebral than in peripheral arteries, as observed by Lee et al. (1980) in the rabbit. A close neuromuscular distance has been suggested to be critical for cocaine-induced prejunctional supersensitivity (Verity 1969).

When the influence of the neuronal uptake mechanism had been reduced by cocaine, NA was approximately 2.5 times more potent in the rat mesenteric artery than in the rat middle cerebral artery. This finding together with the smaller intrinsic activity of NA in the middle cerebral artery may reflect differences between the vessels in the α -adrenoceptor density and/or in the coupling between the receptors and the cellular response.

Studies on pithed rats have indicated the existence of both α_1 - and α_2 -adrenoceptors postjunctionally in VSM (Timmermans et al. 1979, Docherty & McGrath 1980, Kobinger & Pichler 1980, Gerold & Heusler 1983). However, the distribution of these receptors within the rat vascular system has not been established. The present in vitro study (V) suggests that the contractile response to NA in rat middle cerebral and mesenteric arteries is mediated by predominantly α_1 -adrenoceptors. This conclusion is based mainly on the following observations. The selective α_2 -

adrenoceptor agonist clonidine failed to elicit contraction in concentrations lower than 3×10^{-4} M. The selective α_1 -adrenoceptor agonist (-)-phenylephrine, on the other hand, was equipotent with ($\pm$)-NA in the middle cerebral artery and only about two times less potent than ($\pm$)-NA in the mesenteric artery. This agrees well with the range of potency differences suggested by Starke (1981) to be characteristic of α_1 -adrenoceptors.

Oxymetazoline, on the other hand, behaved as a partial agonist in the rat middle cerebral artery with a relatively high potency. This drug has previously been reported to act selectively on α_2 -adrenoceptors (Starke et al. 1975, Docherty & McGrath 1980). Unexpectedly, oxymetazoline also contracted the rat basilar artery, which usually failed to respond to the other α -adrenoceptor agonists. This suggests that oxymetazoline has additional sites of action, unrelated to α -adrenoceptors, and therefore may be an unreliable tool in the classification of α -adrenoceptors in functional studies.

As pointed out by Starke (1981), classification of α -adrenoceptors by means of subtype selective agonists is less straightforward than by using selective antagonists, due to differences in efficacy between agonists. In the present study (V), the selective α_1 -adrenoceptor agonist prazosin was between three and four orders of magnitude more potent than the selective α_2 -adrenoceptor antagonist rauwolscine in inhibiting NA-induced contractions in rat middle cerebral and mesenteric arteries. The experimental results suggest that the inhibition produced by prazosin was of the competitive type in both arteries. The pA_2 ($-\log K_B$) values determined for prazosin in rat middle cerebral and mesenteric arteries largely agree with those reported by others for the interaction between prazosin and α_1 -adrenoceptors in both functional and binding studies (see Bylund & U'Prichard 1983, Steen et al. 1984).

In the rat basilar artery, NA usually failed to elicit contraction even in the presence of propranolol and cocaine, suggesting that this artery lacks contraction-mediating α -adrenoceptors. This interpretation is supported by binding studies demonstrating a paucity of α_1 -adrenoceptors in this preparation,

as compared with the rat tail artery (Silverberg et al. 1982). Thus, in this respect the rat basilar artery differs from the rat middle cerebral artery, indicating regional differences in the α -adrenoceptor distribution in the rat cerebrovascular bed. Notably, the density of adrenergic nerves has similarly been reported to differ between the major cerebral arteries of the rat, the middle cerebral artery being more richly supplied with adrenergic nerves than the basilar artery (Kobayashi et al. 1981).

In agreement with the present study, Hirst et al. (1982) found that exogenously applied NA failed to elicit contraction in the rat basilar artery. However, NA released by electrical field stimulation induced excitatory junctional potentials, sometimes associated with the appearance of a spike potential and contraction (Hirst et al. 1982). Since the excitatory junctional potentials were resistant to inhibition by classical adrenoceptor blocking agents, it was postulated that the response was mediated by a new type of adrenoceptor, termed γ -receptor, present only in the adrenergic neuroeffector region (Hirst et al. 1982).

Involvement of different Ca^{2+} pools in α_1 - and α_2 -adrenoceptor mediated contractile responses

There is ample evidence that NA can induce contraction in several VSM preparations by mobilizing Ca^{2+} from different Ca^{2+} sources (Somlyo & Somlyo 1968, Bolton 1979, Webb & Bohr 1981). The fast component and the slow component of the NA-induced contraction, in e.g. the rabbit aorta and ear artery, have been associated with intracellular Ca^{2+} release and Ca^{2+} influx, respectively (Steinsland et al. 1973, Deth & van Breemen 1974, 1977). Much interest has recently been focused on the Ca^{2+} pools mobilized for contraction after α_1 - and α_2 -adrenoceptor stimulation in VSM. Based on the effects of organic Ca^{2+} entry blockers on vasopressor responses elicited by subtype selective α -adrenoceptor agonists in different animal species, it was suggested that α_1 - and α_2 -adrenoceptors are coupled specifically to intracellular Ca^{2+} release and Ca^{2+} influx, respectively (see van Meel 1982).

In the present study (VI), exposure to Ca^{2+} -free medium consistently reduced the tonic component of the contractile response to NA in rat and cat middle cerebral and mesenteric arteries. A comparison between the effects of Ca^{2+} deprivation on K^{+} - and NA-induced contractions (VI), showed that Ca^{2+} removal more markedly reduced the contractile response to K^{+} than that to NA in rat and cat mesenteric and rat middle cerebral arteries, but not in cat middle cerebral arteries. These results suggest that NA, in addition to mobilizing extracellular Ca^{2+} , also can release Ca^{2+} from a relatively tightly bound Ca^{2+} pool (presumably of intracellular origin) in each type of vessel excluding the cat middle cerebral artery. Thus, NA-induced contractions in rat middle cerebral and mesenteric arteries (supplied predominantly with α_1 -adrenoceptors; V) and contractions elicited by NA in the cat mesenteric artery (sensitive to both α_1 - and α_2 -adrenoceptor selective antagonists; Skärby et al. 1983) appear to depend on both Ca^{2+} influx and intracellular Ca^{2+} release, whereas those in the cat middle cerebral artery (supplied with mainly α_2 -adrenoceptors; Medgett & Langer 1983, Skärby et al. 1983) seem to rely almost entirely on Ca^{2+} influx. This does not support the view that α_1 -adrenoceptors are linked specifically to intracellular Ca^{2+} release, which agrees well with recent studies showing that stimulation of postjunctional α_1 -adrenoceptors in the isolated rabbit aorta and ear artery can induce both Ca^{2+} influx and intracellular Ca^{2+} release (Cauvin et al. 1982, Awad et al. 1983, Manzini et al. 1983). However, our results (VI) do not exclude the possibility of a general lack of coupling between α_2 -adrenoceptors and intracellular Ca^{2+} release, as suggested by, e.g., van Meel (1982).

Regional differences in the excitation-contraction coupling process

Several comparative studies have shown that agonist-induced contractile responses are more sensitive to Ca^{2+} removal and different Ca^{2+} -entry blockers in cerebral arteries than in peripheral arteries (Allen & Banghart 1979, Shimizu et al. 1980, McCalden & Bevan 1981, Towart 1981, Towart & Perzborn 1981, Bevan 1983, Yamamoto et al. 1983). The present study provides further evidence of differences in the excitation-contraction coupling

process between cerebral and peripheral arteries. First, NA-induced contractions were regularly more resistant to Ca^{2+} deprivation in mesenteric arteries than in middle cerebral arteries from both rat and cat, a difference which could be related neither to the size of the vessels (VI) nor to the α -adrenoceptor subtype stimulated by NA. Rather, this suggests that the NA sensitive intracellular Ca^{2+} pool is larger in mesenteric arteries than in cerebral arteries. Secondly, the contractile response to NA was considerably more inhibited by nifedipine in rat and cat middle cerebral arteries than in rat and cat mesenteric arteries. This was true for both the maximum response and the tonic component of the NA-induced contraction. The selectivity for cerebral arteries displayed by nifedipine may reflect differences between the vessels not only in the relative contribution of Ca^{2+} influx and intracellular Ca^{2+} release to the contractile activation, but also in the nifedipine sensitivity of the Ca^{2+} entry pathway utilized by NA.

Interestingly, there appeared to be a correlation between the maximum inhibition of the tonic component produced by nifedipine and the amplitude of the contraction elicited by NA in Ca^{2+} -free medium. This may indicate that the nifedipine resistant part of the tonic component is somehow related to the extent to which NA liberates intracellular Ca^{2+} , i.e. the greater the intracellular Ca^{2+} release, the greater the nifedipine resistance of the tonic response. Such a correlation would be anticipated if the nifedipine resistant part of the tonic component reflects a fast refilling of the NA sensitive intracellular Ca^{2+} store directly from the extracellular medium and subsequent release of Ca^{2+} into the myoplasm. Casteels and Droogmans (1981) have previously suggested that such a pathway, resistant to Ca^{2+} entry blockers, may represent an alternative model of the ROC.

Electrophysiological studies on cat cerebral (Harder et al. 1981) and rat mesenteric (Mulvany et al. 1982) arteries have shown that exposure to 10^{-5} M NA induced a significant membrane depolarization. Since this may lead to activation of POCs, it cannot be excluded that the nifedipine sensitive component of the NA-induced contractions in these arteries was attributed to Ca^{2+} influx through POCs.

SUMMARY AND CONCLUSIONS

A sensitive method for recording of mechanical activity in isolated blood vessels with calibres down to 100 μm was developed. This technique caused no damage to the smooth muscle or endothelial cells, as observed by light, transmission and scanning electron microscopy. It appears that the present in vitro method can be of great value in studies of the VSM function in small blood vessels.

By studying the length-tension relationship in relaxed and K^+ -activated rat basilar arteries, the optimum resting wall tension for recording of contractile activity was determined. The mechanical behaviour of the vessels conformed to the sliding filament theory of muscle contraction. The maximum active wall stress developed by the K^+ -activated arteries was comparable with that found in other VSM preparations.

High K^+ depolarizing medium induced a biphasic contractile response in isolated rat basilar and mesenteric arteries (after elimination of the adrenergic contribution). The hypothesis is put forward that the biphasic course of the contractions reflects influx of Ca^{2+} through two different types of POC, differing in their kinetic properties.

Nifedipine, in concentrations which produced a maximum inhibition of the contractile response to K^+ , failed to significantly alter the K^+ -induced efflux of ^3H from isolated rabbit basilar and facial arteries preincubated with ^3H -NA. The selective action of nifedipine on depolarization-induced contraction over transmitter release may indicate that POCs in peripheral adrenergic nerve endings are different from those in VSM.

It is suggested that K^+ and NA utilize partly different Ca^{2+} entry pathways to increase the myoplasmic Ca^{2+} concentration in isolated rat mesenteric arteries. Whereas K^+ seems to promote the influx of Ca^{2+} by activating POCs, the nature of the receptor-regulated pathway remains to be established.

By means of subtype selective α -adrenoceptor agonists and antagonists, the postjunctional α -adrenoceptors in rat middle cerebral and mesenteric arteries were characterized. The results suggest that the α -adrenoceptors mediating the contractile response to NA in these vascular preparations are predominantly of the α_1 -type. Notably, NA usually failed to elicit contraction in the rat basilar artery, indicating regional differences in the α -adrenoceptor density or function in the rat cerebrovascular bed.

Contractions induced by stimulation of postjunctional α_1 -adrenoceptors in rat middle cerebral and mesenteric arteries appeared to be caused by both Ca^{2+} influx and intracellular Ca^{2+} release, whereas α_2 -adrenoceptor-mediated contractile responses in the cat middle cerebral artery seem to rely almost entirely on influx of extracellular Ca^{2+} . These results do not support the view that α_1 - and α_2 -adrenoceptors are linked specifically to either of these processes, although the possibility of a general lack of coupling between α_2 -adrenoceptors and intracellular Ca^{2+} release cannot be excluded.

NA-induced contractions in cerebral arteries from both rat and cat were generally more sensitive to Ca^{2+} deprivation and nifedipine than were those in mesenteric arteries. This presumably reflects differences between the vessels in (1) the relative contribution of Ca^{2+} influx and intracellular Ca^{2+} release to the contractile activation and (2) the nifedipine sensitivity of the Ca^{2+} entry pathway utilized by NA. Thus, the mechanism through which NA induces contraction in VSM seems to be related not only to the subtype of α -adrenoceptor stimulated by NA, but also to the anatomical origin of the vessel.

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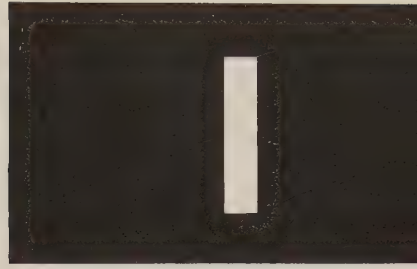
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Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels*

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A sensitive device for recording of mechanical activity in isolated small blood vessels with calibres down to 100 μm is described. This equipment was used to examine the mechanical properties of rat cerebral arteries. The ultrastructure of the preparations was investigated by light-, transmission, and scanning electron-microscopy. In general the walls of the middle cerebral and basilar arteries consisted of 3 layers of smooth muscle cells, which occupied approximately 80% of the total wall thickness. The present technique preserved the integrity of the vessel wall and caused no observable damage to the smooth muscle or endothelial cells. Neither the basilar nor the middle cerebral arteries developed spontaneous phasic contractions under standard conditions. Potassium excess (124 mM) induced a biphasic contractile response characterized by a fast and partly transient increase in tension (phase A), followed by a slowly developing sustained contraction (phase B). The responses to K^+ were strong, highly reproducible and not influenced by pH changes in the range 6.9–7.8, making K^+ -stimulation suitable for testing of vascular contractility. Length-tension measurements were performed on relaxed and K^+ -activated basilar arteries. The mechanical behaviour of the vessels conformed to a sliding-filament model of muscular contraction. Using the "Maxwell model" of a muscle, the length at which the contractile element produced maximum active tension was established. The passive wall tension at this length (~ 1 mN/mm) averaged only about 20% of the total wall tension the arteries were capable of producing when activated by K^+ . Under isometric conditions the K^+ -contracted basilar artery developed a maximum active wall stress of approximately 240 mN/mm². In the light of the mechanical data obtained from the length-tension measurements, the optimum resting wall tension for registration of vascular responses is discussed. It appears that the present in vitro system can be of great value in investigations of the smooth muscle function in small blood vessels.

Key words: Vascular smooth muscle, rat cerebral arteries, potassium contraction, length-tension relation

The brain is for an optimum function critically dependent on an adequate supply of primarily oxygen and glucose via the blood stream. The blood flow to the brain is largely regulated by calibre changes of cerebral arteries and arterioles. Consequently, the mechanical properties of these vessels and the various factors involved in the regulation of their degree of contraction will ultimately determine the responsiveness of the cerebrovascular bed. Although the rat has been frequently used to study

various aspects of cerebral circulation and metabolism in vivo (see Edvinsson & MacKenzie 1977, Siesjö 1978), only fragmentary information exists on the mechanical properties and activation mechanisms of isolated rat cerebral arteries. One reason for this has been the lack of suitable methods to

* This investigation was presented in part at the international symposium "Cerebral Microcirculation and Metabolism" 1980 (Högestätt et al. 1981).

examine small (outer diameter $<400\text{ }\mu\text{m}$) blood vessels *in vitro*. Instead, the mechanical reactivity of rat cerebral vessels has been inferred from investigations on isolated pial arteries from larger mammals such as cats (Edvinsson & Owman 1974), dogs (Allen et al. 1974, Toda et al. 1978), rabbits (Duckles & Bevan 1976), goats (Urquilla et al. 1975), cows (Cheng & Shibata 1978), and man (Edvinsson et al. 1976). This prompted us to develop a modified *in vitro* system, which would enable us to examine the smooth muscle function in small blood vessel preparations.

In the present paper we will describe a sensitive and reliable technique, allowing studies of mechanical activity in isolated blood vessels with calibres down to $100\text{ }\mu\text{m}$. Although the fundamental principles on which the method is based are currently applied in many laboratories, the technical equipment has been improved and specially designed in order to eliminate the difficulties normally encountered in the handling of small vessels. By the use of this method the mechanical properties of isolated rat cerebral arteries were characterized. A morphological analysis of the same vessels were also performed using light-, transmission, and scanning electron-microscopic techniques.

MATERIALS AND METHODS

Adult Sprague-Dawley rats of either sex (weighing 200–300 g) were decapitated, the skull opened, and the brain and medulla rapidly removed and placed in a chilled (12°C) bicarbonate based standard Na^{+} -Krebs solution (for composition, see below). The basilar and middle cerebral arteries were subsequently dissected free from the underlying brain tissue using a dissection microscope. Care was taken not to stretch or otherwise damage the vessels. The arteries were used immediately after sacrifice of the animals unless differently stated. The specific sections of the middle cerebral artery (MCA) and basilar artery (BA) used in the present experiments are indicated in Fig. 1. The outer diameters of the MCA and BA (*in situ*) in these regions were approximately $110\text{ }\mu\text{m}$ and $160\text{ }\mu\text{m}$, respectively.

Recording of mechanical activity

The vessels were divided into 1–2 mm long segments, and suspended in a double mantled small volume (2.5 ml) muscle bath made of Perspex (Fig. 2). Two small stainless steel wires (diameter = $50\text{--}100\text{ }\mu\text{m}$) were gently inserted into the vessel lumen under microscope (Fig. 2). The thin wires were supported by relatively thick metal tubes (outer diameter = 1.2 mm). When the specimen holders were manufactured each wire was bent 90° and tightly attached to the tube. The mechanical activity of the arterial seg-

ment was recorded ‘‘isometrically’’ by a Grass Instrument FT 03C force-displacement transducer connected to one of the two L-shaped specimen holders. The output from the transducer was displayed on a Grass Instrument model 7D polygraph. The opposite specimen holder was attached to a movable unit, permitting precise adjustments of the resting tension. The experimental equipment allowed forces as small as 0.05 mN to be accurately measured. The overall compliance of the recording system, determined by measuring the distance between the steel wires before and after a vessel was contracted, was $5\text{--}10\text{ }\mu\text{m}/\text{mN}$. The two wires remained parallel also after activation of the vessel. During a 60 min equilibration period the resting tension was adjusted to between 1.5 and 2.5 mN (depending on the type and length of the vessel segment to be examined). The temperature was maintained at 37°C by a thermoregulated water circuit heating the organ bath. A mixture of CO_2 (5%) and O_2 (95%) was continuously bubbled through the buffer solution in the organ bath, resulting in a pH of approximately 7.4 (checked by a Radiometer PHM82 standard pH-meter). The bath fluid was routinely changed every 15 min and replaced by fresh buffer (also kept at 37°C and bubbled with 5% CO_2 and 95% O_2).

Length-tension measurements

The caudal half of the rat BA was divided into two segments of equal length (1–2 mm). One of the vessel segments (randomly chosen) was immediately suspended in standard Na^{+} -Krebs solution and activated by a K^{+} rich buffer solution (for composition, see below). Following a short accommodation period (10 min), the distance between the steel wires was slowly increased, each time by $35\text{ }\mu\text{m}$, and the tension recorded at different internal diameters. The tension was always allowed to reach a steady level in between the extensions. The magnitude of this steady state tension was used in the calculations. The adjacent vessel segment was transferred into a Ca^{++} deficient Na^{+} -Krebs buffer (for composition, see below) and stored in this solution for between 3 and 24 h at 12°C in order to deplete the smooth muscle cells of activator calcium, and thereby reduce the activity of the contractile system to a minimum. The relaxed arterial segment was subsequently suspended in this Ca^{++} free solution, and subjected to stretches as described above. After mounting, all vessel segments were subjected to a small preload ($\sim 0.2\text{ mN}$) from which they were allowed to relax for 60 min before the various procedures described above were commenced.

Theoretical considerations

With the vessels mounted in the muscle bath, the following parameters were measured by means of a dissection microscope (Wild M3, equipped with a scaler) placed immediately above the organ bath (magnification $\times 120$, resolution $5\text{ }\mu\text{m}$); wire thickness (d), distance between the outer limits of the wires (f), vessel length (e). Since the vessel wall was stretched between the wires the internal circumference (L) was estimated as follows:

$$L = 2f + d(\pi - 2) \quad (1)$$

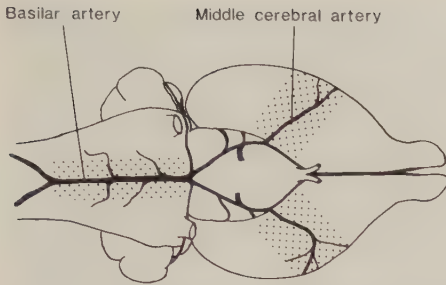


Fig. 1. View of the major pial arteries on the base of the rat brain. The particular segments of the basilar and middle cerebral arteries selected for the experiments are enclosed in the dotted areas.

The effective lumen radius (ELR) was then determined on the basis of the computed internal circumference (Mulyan & Halpern 1977):

$$\text{ELR} = \frac{L}{2\pi} \quad (2)$$

In order to relate the force (F) recorded by the force-displacement transducer to the circumferential wall tension (WT), the following formula was used:

$$\text{WT} = \frac{F}{2e} \quad (3)$$

The calculation of the active wall tension (ΔWT) was based simply on a subtraction of the mean WT recorded in relaxed vessels (passive WT, WT_p) from that recorded in K^+ -activated vessels (WT_a) for each ELR. Thus, the change in WT upon activation was:

$$\Delta\text{WT} = \text{WT}_a - \text{WT}_p \quad (4)$$

The active wall stress (ΔWS) was derived from the ΔWT and the wall thickness (h). This parameter expresses the tangential ΔWT per unit wall thickness or force per unit area:

$$\Delta\text{WS} = \frac{\Delta\text{WT}}{h} \quad (5)$$

Assuming that the vessel wall was incompressible and that the length of the arterial segment (e) remained constant, the wall thickness at the various vessel diameters was obtained from the following equation:

$$h = \sqrt{\frac{A}{\pi} + \text{ELR}^2} - \text{ELR} \quad (6)$$

where A is the mean cross section area of the vessel wall determined from light microscopic photomicrographs (corrections for artifacts introduced by the fixation procedure were not made).

The effective transmural pressure (ETP) was computed from Laplace's equation given a measured WT and ELR:

$$\text{ETP} = \frac{\text{WT}}{\text{ELR}} \quad (7)$$

The calculated ETP was considered only as an approximate value of the maximum intravascular pressure that a particular vessel would resist or produce at a certain lumen radius and degree of activation. In other words ETP describes the transmural pressure that would have been required at the different internal diameters to balance the forces in the vessel wall (be they passive or active), which strive to constrict the vessel.

Response to potassium at different pH levels

These experiments were not continued unless at least two reproducible contractions induced by a 124 mM K^+ solution (for composition, see below) were first obtained. Contractions were considered reproducible when the maximum tensions differed by less than 5%. A pH of 7.80 ± 0.05 in the organ bath was achieved by increasing the NaHCO_3 concentration in the buffer solutions from 15 to 30 mM, and a pH of 6.90 ± 0.05 by decreasing the NaHCO_3 content from 15 to 5 mM (corrections for changes in tonicity were not made). The arteries were always left to accommodate in their new milieu for 30 min before contractions were induced by K^+ . The pH was

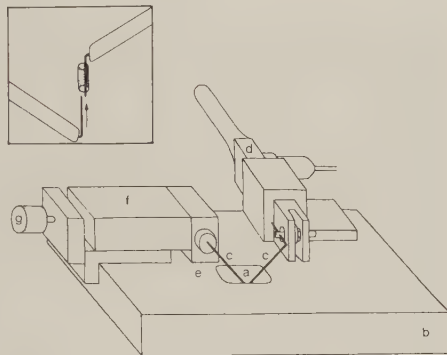


Fig. 2. Schematic drawing of the myograph. Muscle bath (a). Myograph block (b). Two small stainless steel wires (diameter = 50–100 μm), attached to the distal ends of the specimen holders (c), were gently passed into the blood vessel lumen (see inset). One of the specimen holders was connected to a Grass Instrument FT 03 C force-displacement transducer (d) and the tension in the vessel wall monitored on a Grass Instrument polygraph. The opposite specimen holder was fixed to a small cylinder (e), freely movable inside a displacement unit (f). The latter unit was manipulated by a micrometer screw (g), and used to adjust the resting tension in the preparation. A gas mixture was conveyed through a small tubing, entering at the back of the Perspex block, to the bottom of the muscle bath. Heated water, delivered by a thermostatically controlled pump unit, was continuously circulating around the muscle bath inside the myograph block.

continuously registered by a small pH-electrode (see above) placed in the organ bath.

Light and electron microscopy (performed at the Electron Microscopy Laboratory of the Oncology Clinic, University Hospital of Lund)

Pieces of the MCA and BA were suspended between the steel wires in the muscle bath, as described above, and subjected to resting WTs between 0.25 and 2.5 mN/mm. After an accommodation period of about 60 min in standard Na^+ -Krebs buffer, the preparations were exposed to 3% glutaraldehyde in 0.15 M cacodylate buffer (prewarmed to 37°C), containing 10 mM CaCl_2 (pH=7.3–7.4). A few arterial segments were contracted by 124 mM K^+ before fixation. The vessels were gently removed from the specimen holders after 30 min, and the glutaraldehyde fixation continued for another 2 h. Another series of arteries were detached from the wires before fixation. Control vessels were not suspended in the organ bath but immersed into the fixative immediately after excision. Following fixation, the arterial segments were extensively rinsed in glutaraldehyde free cacodylate buffer.

Tissues for transmission electron microscopy were postfixed in 1% osmium tetroxide, dehydrated in ethyl alcohol, and subsequently embedded in Epon. Sections of 1–2 μm thickness were examined in a light microscope after staining with toluidine blue. Ultrathin sections were cut on a LKB-ultratome, mounted on copper grids, and contrasted with uranyl acetate (0.5%) and lead citrate (1%). The sections were viewed in a Zeiss EM10 electron microscope.

Specimens for scanning electron microscopy were dehydrated in graded ethyl alcohol, and exposed to freon overnight. They were dried according to the critical point method with CO_2 , and finally coated with gold-palladium in a vacuum evaporator by ion sputtering. The specimens were examined in a Cambridge MK II Scanning Electron Microscope.

Buffer solutions

1. The standard Na^+ -Krebs solution (NaKs) was composed of (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, glucose 6.

2. The Ca^{++} free solution consisted of the same constituents as the NaKs solution apart from the following changes; the CaCl_2 was omitted and replaced by 0.01 mM bis-(aminoethyl)-glycoether-N,N,N',N'-tetraacetic acid (EGTA).

3. The isotonic K^+ -Krebs solution (KKs) contained 124 mM K^+ , and was prepared by exchanging the NaCl in the NaKs for KCl in equimolar amounts.

Analytical grade chemicals and redistilled water were used for preparing all solutions.

Drugs

The drugs used were histamine hydrochloride (Sigma Chemical Company), isoprenaline hydrochloride (Riker), and nifedipine (Adalat®, Bayer AG). Nifedipine was provided in ampoules (0.1 mg/ml) and kept dark until used to avoid light-induced degradation.

The drugs were dissolved and/or diluted in 0.9% NaCl

(containing 1 mM ascorbic acid), and added directly to the organ bath in volumes of 25 μl . Fresh dilutions of the drugs were made daily. The concentrations reported are the final bath concentrations.

Statistics

Statistical analysis of the results was performed by using Student's *t*-test for paired or unpaired data when appropriate. The 0.05 level of probability was accepted as significant. The results shown in tables, figures and the text are expressed as the mean value $\pm$ standard error of the mean.

RESULTS

Morphology

Light- and transmission electron-microscopy. Generally, the walls of the rat MCA and the BA consisted of 3 layers of smooth muscle cells arranged circumferentially (Fig. 3). About 75–80% of the total wall thickness was occupied by the muscular layer. The smooth muscle cells contained, besides their elongated nuclei, numerous mitochondria. These were found in all parts of the muscle cells, but were most abundant in the perinuclear regions. The endothelial cell layer was separated from the media by a subendothelial zone (Fig. 3). A complex network of collagenous filaments was observed in the adventitia and in coherent arachnoid membranes. Collagenous filaments were occasionally found in the interstitial space in between the smooth muscle cells. The wall thickness of the BA suspended at a resting WT of 1 mN/mm (corresponding to a distending pressure of approximately 50 mmHg) was $20 \pm 3 \mu\text{m}$ ($n=5$), giving a wall thickness to internal radius ratio of about 1/8. The deformation of the arterial wall imposed by the wires was considerably increased with decreasing wire diameter. This was particularly evident in K^+ -contracted arteries. We therefore considered it preferable to use as large wires as was technically possible without causing damage to the preparations when they were set up in the muscle bath. Although the individual smooth muscle cells appeared slightly thicker in K^+ -activated arteries, the wall thickness and the number of muscle cell layers were not appreciably altered. In most of the cases the constituents of the arterial wall, including the endothelial cell lining, displayed normal staining properties and were ultrastructurally intact. This was true also for the portion of the vessel wall which had been in contact with the wires.

Scanning electron microscopy revealed a con-

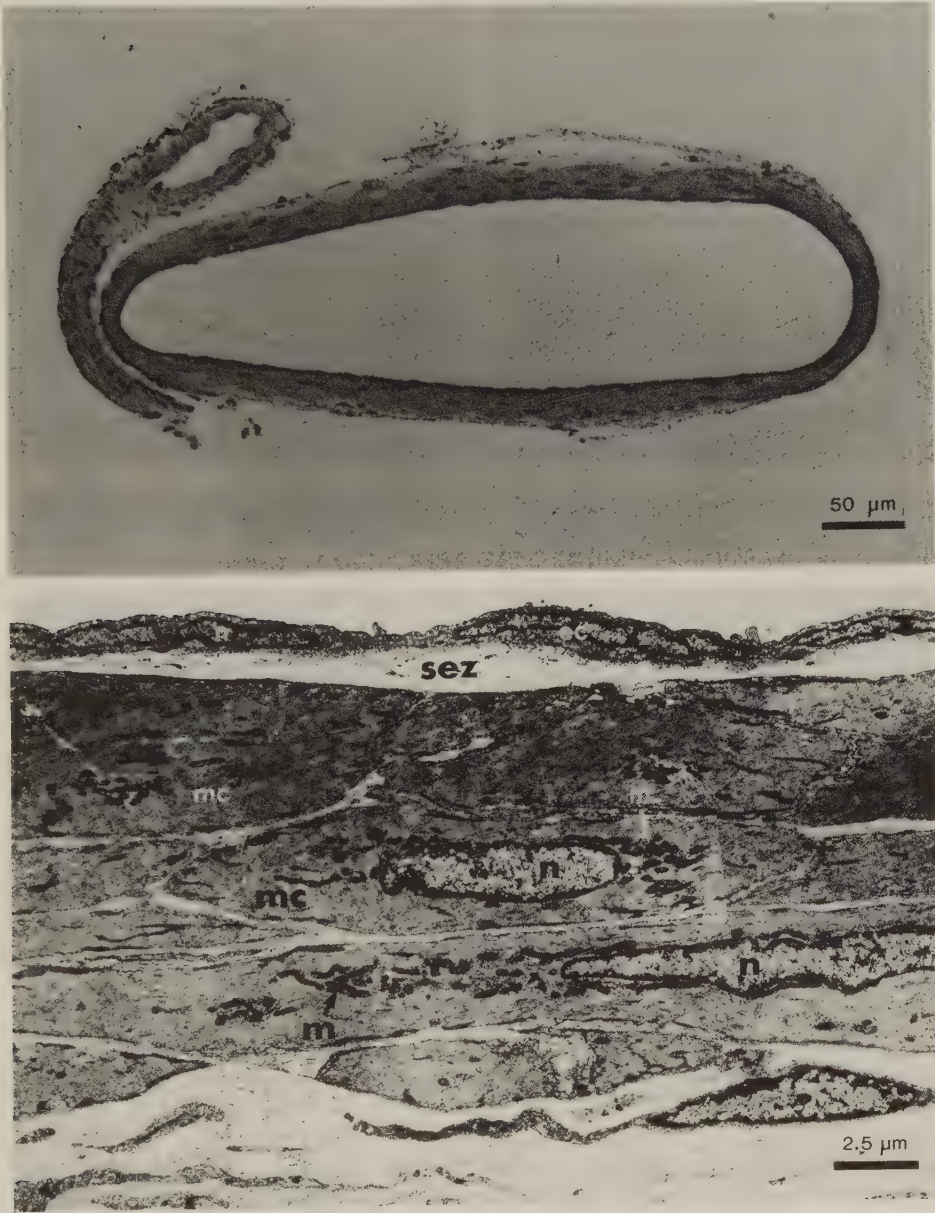


Fig. 3. Upper: Light microscopy of a cross sectioned rat basilar artery. The vessel was subjected to a resting wall tension of about 1 mN/mm, and subsequently glutaraldehyde fixed in the muscle bath. Lower: Photomicrograph showing the ultrastructure of the vessel wall, as demonstrated by transmission electron microscopy (cross section). ec = endothelial cell, mc = muscle cell, sez = subendothelial zone, n = nucleus, m = mitochondria.

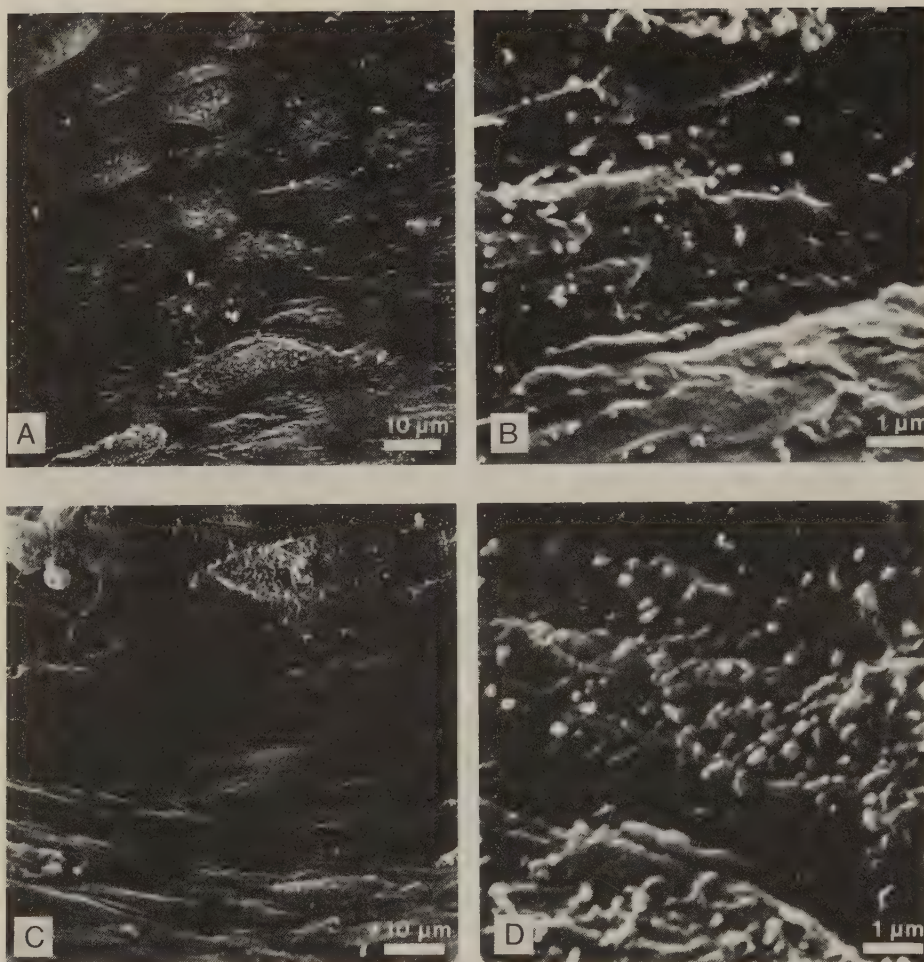


Fig. 4. Scanning electron microscopy of the endothelial cell surface of a rat basilar artery. The vessel was incubated in the muscle bath for 60 min under a resting wall tension of approximately 1 mN/mm, and then removed from the specimen holders and glutaraldehyde fixed. All photographs are from the same arterial segment. The upper two photographs (A and B) show the surface structure of an area which was situated between the wires. A region of the vessel wall which was in contact with one of the wires is shown in C and D. Numerous microvilli were observed in all parts of the endothelium at high magnification (B and D).

tinuous and well preserved endothelial cell surface in all specimens examined. In control arteries (not suspended before glutaraldehyde fixation) the surface appeared pleated with several infoldings running parallel to the axis of the vessel segment. These infoldings were less prominent and the sur-

face structure appeared more smooth in arterial segments stretched between the wires before fixation. At high magnification all surface areas appeared intact with numerous pits and microvilli protruding from the endothelial cells (Fig. 4). A small impression of the endothelium at the site of

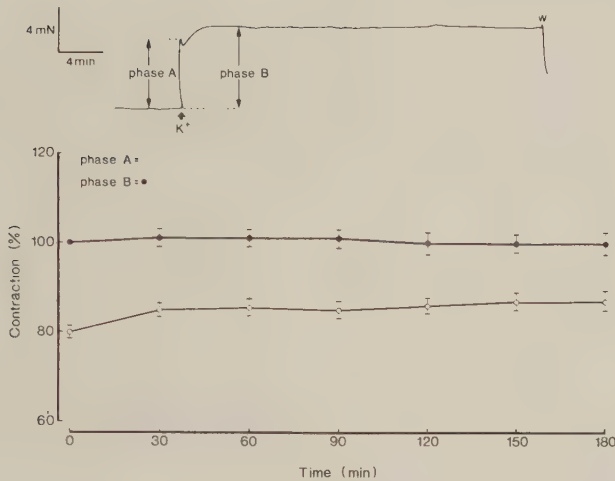


Fig. 5. Stability and reproducibility of K^+ -evoked contractions in isolated rat basilar arteries. The two phases of the K^+ contraction are indicated in the upper tracing ('isometric' recording). The amplitudes of the two components following repeated application of 124 mM K^+ are depicted in the lower diagram. Phase B was taken as the magnitude of the contraction 5 min after the introduction of K^+ , as the response was usually stable at this time. The first phase B response was set to 100%. w=wash.

the steel wires was observed in arteries fixed while remaining suspended in the muscle bath.

General mechanical observations

With the MCA and the BA suspended in the muscle bath, stretches invariably resulted in an immediate increase in tension followed by a rapid relaxation of the vessel segments. The tension stabilized after 5 to 15 min. The magnitude of the response was related to the rate of the length change; the more rapid the stretch the larger the tension increase. Such a viscoelastic behaviour in response to stretches is generally observed in most vascular smooth muscle (VSM) tissues (Somlyo & Somlyo 1968, Dobrin 1978). Ca^{++} withdrawal and exposure to vasodilators such as nifedipine (0.3 μ M) and histamine (0.1 mM) failed to affect the tension of the resting BA. However, these procedures frequently induced a minor relaxation in the MCA.

An artery purposely damaged (e.g. by pinching the vessel wall with a pair of forceps) displayed a different pattern of response. When such vessels were subjected to stretches, tonic contractions developed, and the preparations failed to relax properly. These contractions could be reversed by nife-

dipine (0.3 μ M), histamine (0.1 mM), and by exposing the vessels to Ca^{++} free medium.

Response to potassium

The isotonic K K s elicited strong contractions in the MCA and the BA, and the responses could be divided into two distinct components (Fig. 5). With less than 1 s latency K^+ induced a rapid increase in tension (phase A), and after a small intervening relaxation a sustained tonic contraction developed (phase B). This tonic component was always larger than the rapid one. Phase A of the K^+ contracture arrived at its peak tension within 10 s after introduction of K^+ , while the tonic component required approximately 5 min to be fully developed. The tonic contraction then remained stable for at least 60 min (periods longer than 60 min were not tested). When the K K s was removed and replaced by fresh Na K s the vessels immediately relaxed. The K^+ -induced contraction could usually be repeated after 10 min without a significant reduction of the maximum tension. Repeated application of K^+ every 30 min for 3 h produced highly reproducible responses. However, the amplitude of phase A of the first K^+ contraction was significantly lower

Table 1. Influence of the hydrogen ion concentration on the biphasic vasoconstrictor response to K^+ (124 mM) in the isolated rat basilar artery

The pH was altered by changing the $NaHCO_3$ concentration in the buffer solutions (see Methods). The amplitude of phase B of the K^+ contraction immediately preceding each pH change was taken as 100%. For definition of the two components of the K^+ contraction, see Fig. 5. n=number of arteries examined (all from different animals)

pH	n	Phase A (%)	Phase B (%)	Phase B/Phase A
7.4 (control)	7	86 ± 1.5	100	1.17 ± 0.02
6.9	7	85 ± 1.3	102 ± 0.8	1.20 ± 0.02
7.4 (control)	7	84 ± 1.8	100	1.20 ± 0.02
7.8	7	85 ± 1.7	100 ± 0.4	1.19 ± 0.02

($p < 0.001$) than that of the consecutive responses (Fig. 5). Although the larger part of these experiments were performed on BAs, similar results were obtained with the MCA.

Neither the maximum of phase A and phase B nor the succeeding relaxation occurring on removal of K^+ were changed when the pH of the buffer solutions was varied between 6.9 and 7.8 (Table 1). The time to peak tension of phase A and phase B was also not influenced by the altered hydrogen ion concentrations. However, in some arteries an increment of the pH from 7.4 to 7.8 induced a small sustained contraction.

Length-tension characteristics

Length-tension measurements were performed on relaxed and K^+ -activated BAs from 8 rats. The relationship between ELR and WT was studied over a range of ELR between 75 and 200 μm . The results from these experiments are illustrated in Fig. 6. As can be seen no WT was demonstrated in arteries relaxed in Ca^{++} free medium at ELR smaller than 110 μm . However, beyond this point the WT_p progressively increased and so did the elastic modulus. K^+ activation considerably enhanced the WT in the arterial segments, especially at small ELR (Fig. 6). The ELR-WT curves obtained from relaxed and activated vessels converged at large ELR. Exposure to nifedipine (0.3 μM) or to a Ca^{++} free NaKs failed to reduce the tension of K^+ -activated arteries stretched to ELR larger than 200 μm ($WT > 15$ mN/mm). This would indicate that the WT at these ELR is due almost entirely to passive elements in the vessel wall.

The mean increase in WT upon K^+ activation was calculated for each ELR using Eq. 4. As can be seen in Fig. 7 the ELR- ΔWT curve reached a maximum of 4.8 mN/mm at an ELR of approximately

150 μm and then declined. The WT_p at this ELR (~ 1 mN/mm) averaged only about 20% of the total WT the artery was capable of producing when contracted by K^+ . The intraluminal pressure corresponding to this WT_p (calculated according to the

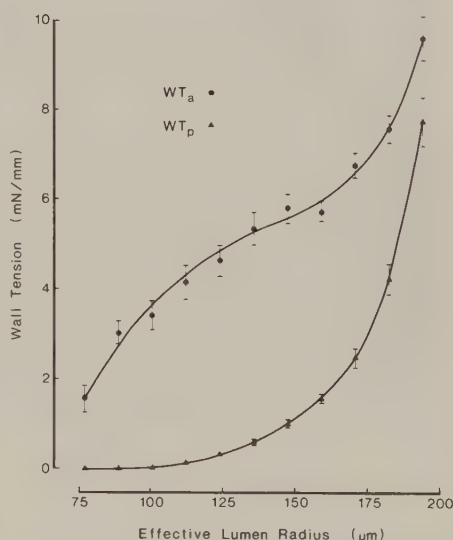


Fig. 6. Relation between effective lumen radius (ELR) and wall tension in relaxed (WT_p , $\blacktriangle$) and K^+ -activated (WT_a , $\bullet$) rat basilar arteries. The WT_p at the various ELR was derived from arteries immersed in ' Ca^{++} free' NaKs. The vessels were slowly extended from slack length to an ELR of about 200 μm and the wall tension measured at predetermined ELR. Separate arterial segments were initially contracted by K^+ before subjected to identical stretches. Each point in the diagram is the mean of 6 to 8 measurements (all on different arterial segments). For further details, see Methods.

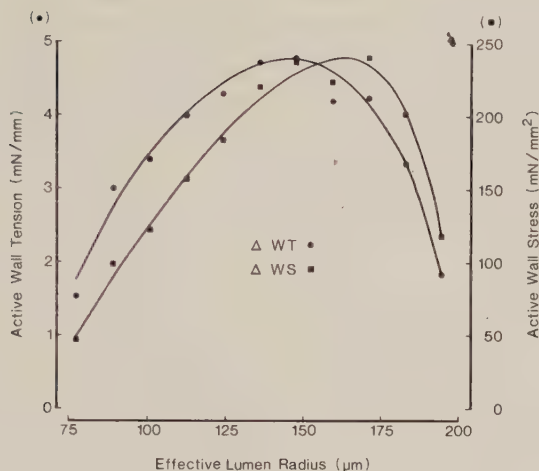


Fig. 7. Dependence of the active wall tension (ΔWT , $\bullet$) and active wall stress (ΔWS , $\blacksquare$) on the effective lumen radius (ELR) in the isolated rat basilar artery. The ΔWT was derived from the mean WT in relaxed and K^+ -activated arteries for each ELR by simple subtraction (Eq. 4). The ΔWS was obtained from the ΔWT and the wall thickness prevailing at that ELR. The wall thickness at the various ELR was computed according to Eq. 6 in Methods. Conditions as in legend to Fig. 6.

law of Laplace) was about 50 mmHg. This is in accordance with the perfusion pressure found by Uchida et al. (1967) to elicit a maximum response to a test dose of noradrenaline in isolated rabbit mesenteric arteries of similar dimension.

The ΔWS was derived from the ΔWT and wall thickness as described in Methods. Since the wall thickness changes when the vessel is extended, this parameter was first computed for each ELR according to Eq. 6. The ΔWS obtained at the various ELR is plotted in Fig. 7 using the same abscissa as for the ΔWT data. It is apparent that the ELR- ΔWS curve and the ELR- ΔWT curve have similar shapes. However, due to the fact that a vessel becomes thinner when stretched, the peak of the ELR- ΔWS curve was obtained at a somewhat larger ELR. The ΔWS at this ELR was approximately 240 mN/mm².

The WT data shown in Fig. 6 have been transferred into terms of ETP in Fig. 8 using Eq. 7. As was expected the ETP_p increased exponentially with the ELR, as did the WT_p . However, the ELR- ETP_a curve showed a plateau at an ETP of approximately 250 mmHg. As mentioned in the method section, we have considered the ETP to describe

the maximum transmural pressure a vessel would resist or produce at a given degree of activation and lumen diameter. Accordingly, the K^+ -activated rat BA would be capable of withstanding intravascular pressures of up to at least 250 mmHg at a fairly wide range of lumen radii ($>90 \mu m$). Since the WT is directly proportional to the internal radius at a constant transmural pressure (Laplace's law), the forces which are required to balance the distending pressure will increase with increasing lumen radius. At small ELR ($90 \mu m < ELR < 150 \mu m$) this was probably achieved by the increase in ΔWT which follows distension of the artery. At large ELR passive WT will progressively develop and compensate for the continuous decay of the ΔWT , occurring at ELR exceeding $150 \mu m$.

DISCUSSION

A multitude of techniques have been utilized for studying mechanical activity in isolated VSM preparations, among these perfusion of intact blood vessels (Lande & Rand 1965, Uchida et al. 1967, Duling et al. 1981), helically cut preparations (Bohr et al. 1961, Toda & Fujita 1973), and methods similar

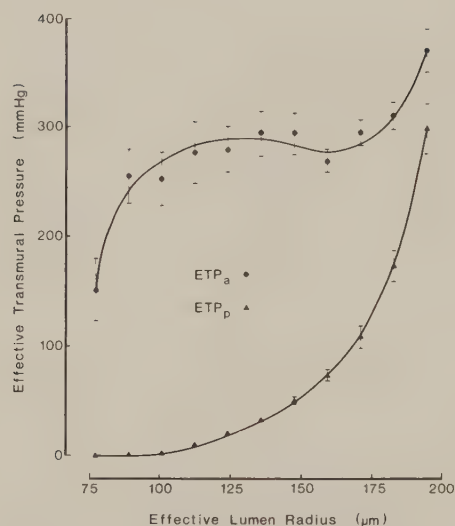


Fig. 8. Effective lumen radius—effective transmural pressure diagrams for relaxed (ETP_p , $\blacktriangle$) and K^+ -activated (ETP_a , $\bullet$) rat basilar arteries. The ETP values were calculated from the wall tension data given in Fig. 6 according to Ep. 7 (Laplace's law). Conditions as in legend to Fig. 6. $n=6-8$.

to the present one (Bevan & Osher 1972, Edvinsson et al. 1974). Each of these techniques has both advantages and disadvantages. With a few exceptions most established *in vitro* methods are confined to relatively large vessels, which are probably not of great hemodynamic importance. The present method allows studies of mechanical activity to be performed on small vessels with lumen radii down to $50\ \mu\text{m}$. Vessels of this size are likely, at least in man, to take active part in the regulation of vascular resistance and capillary pressure (Folkow & Neil 1971). Arterial segments of similar dimension from rat mesentery and rabbit brain have recently been examined in detail using another type of myograph (Mulvany & Halpern 1977, Duckles & Bevan 1979). A sophisticated perfusion technique for true arterioles has also been described (Duling et al. 1981).

The present *in vitro* technique preserved the integrity of the vessel wall and caused no observable destruction of the smooth muscle or endothelial cells, even in the area which wrapped around the wires, as shown by light and electron microscopy.

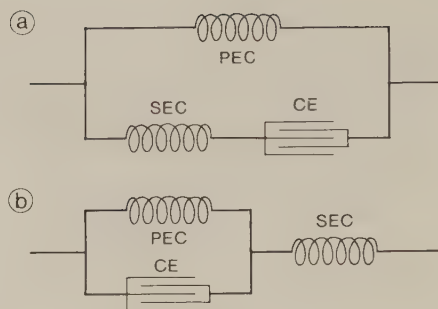


Fig. 9. Two conceptual muscle models generally referred to in muscle mechanics; (a) the "Maxwell model" and (b) the "Voigt model" (see Dobrin 1978). The classical model for muscle contraction proposed by Hill (1938) was composed of only two components, a contractile element (CE) in series with an undamped series elastic component (SEC). However, this model does not account for the resting (passive) tension in a relaxed muscle. In order to do so it is necessary to include a third parallel elastic component (PEC). This latter element can be combined in parallel either with both the CE and the SEC (as in a) or with the CE alone (as in b).

This is important particularly since Furchgott & Zawadzki (1980) have demonstrated the necessity of an intact endothelial cell layer for a proper function of arteries *in vitro*. Furthermore, K^+ excess elicited strong and reproducible contractions for long periods of time, indicating that the isolated arterial preparations were also maintained functionally and metabolically intact.

Also other types of luminal smooth muscle tissues (as well as strip preparations) can be examined with the present myograph by choosing steel wires with suitable diameters for the specimen holders. The recording system proved to be very sensitive, and allowed mechanical forces as small as $0.05\ \text{mN}$ to be accurately measured. Due to the small volume of the organ bath ($2.5\ \text{ml}$) only small amounts of the test drugs will be required to obtain even high concentrations in the bath solution. This is a considerable advantage when the effects of substances available only in small quantities are examined (e.g. peptides, prostaglandins, and leukotrienes). Moreover, the temperature and the pH were easily kept constant in the muscle bath.

The tension in the resting rat BA and MCA was generally not (BA) or only slightly (MCA) altered by Ca^{++} withdrawal and potent vasodilators, sug-

gesting that the smooth muscle cells in the vessel walls are almost entirely relaxed during resting conditions. The length-tension measurements clearly showed that the ability of the arterial wall to develop $\Delta W T$ changes when the vessel diameter is altered. This fundamental feature has been demonstrated in a number of VSM preparations (see Dobrin 1978) including cerebral vessels (Toda et al. 1978, Nagasawa et al. 1979). The shape of the ELR- $\Delta W T$ curve can be readily explained on the basis of a sliding filament model for muscular contraction as originally described for skeletal muscle (see Huxley 1957). Although the contractile protein content and the proportions of actin and myosin filaments in VSM are considerably different from those in striated muscle (Somlyo et al. 1973, Murphy et al. 1974), a large bulk of information favours a sliding filament hypothesis of muscular contraction also for smooth muscle (see Murphy 1976). However, the filament organization and the pattern of sliding may be markedly different from that in striated muscle (Murphy 1979).

In order to accept the ELR- $\Delta W T$ curve for the BA as a true reflection of the capacity of the smooth muscle to develop active tension at different vessel radii, two requirements have to be fulfilled. Firstly, the mechanical behaviour of the arterial segments has to be predictable from a simple muscle model (Fig. 9a), composed of a parallel elastic component (PEC) arranged in parallel with a contractile element (CE) and a series elastic component (SEC). Such a model allows the mechanical output from the contractile machinery to be calculated simply by subtracting the $W T_p$ from the $W T_a$. Theoretically, the PEC may also be combined in parallel with the CE only, as shown in Fig. 9b. A contraction will under these conditions cause a shortening of both the CE and the PEC, and the latter will no longer support the same force as it did at rest. The peak of the ELR- $\Delta W T$ curve will, using this muscle model, merely present the length at which the internal shift of force will conceal any further increase in development of tension by the CE. Although both analogue models have proven useful in analyses of the mechanical behaviour of smooth muscle, the first arrangement has been favoured to describe the elastic and contractile properties of vascular tissues (Dobrin 1978, Mulvany & Warshaw 1979). Secondly, it is required that the degree of activation of the contractile mechanism is constant during the entire course of the length-

tension measurement. The observation that K^+ -induced contractions remained stable for periods longer than the time needed to complete a length-tension recording was taken as an indirect evidence that the arteries did not fatigue during the measurements. Neither did we presume that the activated contractile machinery was damaged when the arteries were subjected to stretches (C. F. Cox 1976), at least not in the range of diameters within which the vessels would normally operate under *in vivo* conditions.

It is reasonable to assume that the optimum vessel radius for detection of vascular responses represents the muscle length at which the overlap between the contractile filaments is optimum. In a recent study on rat mesenteric arteries Mulvany & Warshaw (1979) pointed out that "there is a significant rearrangement of the cells by activation, implying that some lengthen while others shorten". This observation would indicate that the length-tension characteristics of a whole muscle does not necessarily coincide with those of the individual smooth muscle cells. In this context the optimum overlap between the myofilaments will refer to what is optimum for the whole preparation and not to what might be the optimum for the individual smooth muscle cells or sets of interdigitating myofilaments, respectively. In a relaxed vessel the stiffness of the SEC by far exceeds the static stiffness of the CE, and an extension of the preparation will result in an elongation preferentially of the CE. In contrast, the stiffness of the CE in a contracted artery is considerably increased, and stretches will under these conditions cause a relatively larger elongation of the SEC compared to that in a relaxed vessel. The presence of a compliant SEC would also permit the CE to undergo considerable internal shortening even under apparently isometric conditions. Consequently, the actual degree of overlap between the myofilaments at a certain vessel diameter will differ between a relaxed and a contracted artery. Thus, the optimum length for detection of vascular responses will depend on the level of activation, so that in a relaxed artery it would occur at a slightly smaller arterial diameter than it would in the same vessel when contracted. Due to this fact the ELR of a *relaxed* artery, at which the overlap between the myofilaments is optimum, cannot be determined accurately from the length-tension diagrams described above. The ELR at the peak of the ELR- $\Delta W T$ curve and the corresponding passive

WT prevailing at that radius may therefore be used only as approximative values of the optimum vessel radius and resting WT for measuring mechanical activity. Hence, a resting WT of about 1 mN/mm was considered appropriate and used in all subsequent experiments on the rat BA.

It is generally believed that high K^+ solutions stimulate contraction of VSM by increasing the influx of extracellular Ca^{++} (Hudgins & Weiss 1968, van Breemen et al. 1972, Droogmans et al. 1977). Basically the elevated K^+ concentration in the bath fluid will depolarize the muscle membrane due to a shift of the K^+ equilibrium potential towards less negative values. The depolarization is thought to increase the membrane permeability to Ca^{++} , which permits Ca^{++} to move passively along its electrochemical gradient into the cytoplasm and initiate contraction (Bolton 1979, Hagiwara 1981). This ability of K^+ to bypass receptor mediated activation mechanisms makes K^+ stimulation suitable for testing of vascular contractility. In the present study K^+ elicited strong, stable, and highly reproducible contractions in rat cerebral arteries, and as in other types of VSM K^+ induced a sustained contraction (see Bolton 1979). When we examined the contractile effect of a number of different vasoconstrictor agonists such as noradrenaline, serotonin, prostaglandin $F_{2\alpha}$, and angiotensin II on isolated rat pial arteries, it was found that none of these produced a contraction stronger than that evoked by K^+ (unpublished observations). It seems therefore that the maximum response to K^+ would provide a good estimate of the force generating capacity, and it may thus also be used to quantify the amount of functioning smooth muscle in the vessel wall. In fact, the maximum active stress developed by the K^+ -activated rat BA (~ 240 mN/mm²) is comparable with that found in other smooth muscle preparations (see Murphy 1976, Dobrin 1978). However, the WS, WT, and ETP data presented above must be considered with some caution and not directly translated into the *in vivo* situation, since it is known that excised arteries retract to a length slightly smaller than their *in situ* length (Dobrin 1978). As this was not corrected for in the calculations, these parameters might have been slightly overestimated.

It would appear that the present *in vitro* system can be of great value in investigations of the smooth muscle function in small blood vessels. This will give an opportunity to directly compare the me-

chanical reactivity of isolated rat pial arteries with the responsiveness of the rat cerebral circulation *in vivo*. Information of this kind can be useful when the mechanisms underlying the action of various agents on the brain circulation are elucidated.

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**MECHANISMS BEHIND THE BIPHASIC CONTRACTILE RESPONSE TO POTASSIUM
DEPOLARIZATION IN ISOLATED RAT CEREBRAL ARTERIES**

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Running title: K^+ contractions in rat cerebral arteries

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Abstract

Ring segments of rat basilar and middle cerebral arteries were suspended in a small volume muscle bath and the mechanical activity recorded "isometrically". K^+ excess (124 mM) evoked a biphasic contractile response, composed of an early rapid phase (phase A) and an ensuing slow phase (phase B), separated by a small transient relaxation. Cooling produced a gradual dissociation of the two contraction components and depressed their maxima. Readdition of Ca^{2+} to arteries previously depolarized by K^+ in the absence of external Ca^{2+} also elicited a biphasic contraction, which excludes the possibility that the initial transient response was initiated by a burst of spikes. Ca^{2+} removal considerably suppressed and 1 mM La^{3+} abolished both components of the K^+ contraction. Prolonged treatment (>3 hr) in Ca^{2+} -free, EGTA (1 mM)-containing medium reduced neither the amplitude of phase A nor that of phase B of the contraction induced by the simultaneous addition of K^+ and Ca^{2+} . This indicates that the early rapid component was not due to a release of intracellularly stored Ca^{2+} . Nifedipine preferentially inhibited phase B of the K^+ contraction. The drug also effectively suppressed Ca^{2+} -induced contractions in arteries previously depolarized by K^+ in Ca^{2+} -free medium. The inhibition produced by nifedipine consisted of a reduction of both the maximum and the slope of the concentration-response curve for Ca^{2+} . The results of the present study indicate that K^+ initiates contraction in rat cerebral arteries by promoting the movement of extracellular and/or superficially bound Ca^{2+} to the cytoplasmic matrix. It is suggested that the two-component contractile response to K^+ reflects the inflow of Ca^{2+} through two separate K^+ -activated Ca^{2+} channels in the surface membrane, one permitting a rapid and transient movement of Ca^{2+} across the smooth muscle membrane (the "rapidly activated Ca^{2+} channel"), the other allowing Ca^{2+} to pass at a slower rate (the "slowly activated Ca^{2+} channel").

Abbreviations: VSM, vascular smooth muscle; AP, action potential; NA, noradrenaline; NaKs, Na^+ -Krebs solution; BA, basilar artery; MCA, middle cerebral artery; KKs, K^+ -Krebs solution; EGTA, ethylene glycol bis(β -aminoethyl ether)N,N'-tetraacetic acid.

Introduction

Due to the ability of externally applied K^+ to depolarize the muscle membrane, solutions with high K^+ concentrations have been widely employed to study the coupling between membrane depolarization and contraction. In isolated frog skeletal muscle fibers, excess K^+ induces a single transient contracture (Hodgkin and Horowicz, 1960). However, most smooth muscle tissues respond to K^+ with sustained contractions, which usually can be divided into two or more components (for references, see Bolton, 1979). We have previously reported (Högestätt et al., 1983) that isolated rat cerebral arteries exposed to high K^+ solutions display a biphasic contractile response, composed of an initial fast, partly transient contraction (phase A) and an ensuing, more slowly developing sustained contraction (phase B). A similar bimodal appearance of the response to K^+ has previously been observed in intestinal smooth muscle, myometrium, vas deferens, ureter, portal vein and other types of VSM preparations (for references, see Bolton, 1979).

Numerous explanations have been advanced for the various components of the K^+ contraction. In AP discharging smooth muscle tissues, such as the guinea-pig tenia coli and rat ileal smooth muscle, the fast phasic response to K^+ has been attributed to an initial burst of spike potentials (Shimo and Holland, 1966; Imai and Takeda, 1967; Syson and Huddart, 1973). K^+ may also cause neuronal transmitter release and thus indirectly influence the mechanical response via stimulation of receptors at the muscle cell membrane. Such a mechanism was suggested to underlie the large transient relaxation separating the first and second component of the K^+ contraction in uterine strips from oophorectomized rats (Bengtsson, 1977).

A large bulk of information suggests that the sources supplying activator calcium used for contraction may vary not only between different smooth muscle types and between contractions induced by different agents, but also during the course of a single contraction. Thus, it has been proposed that the initial rapid and ensuing tonic component of the NA-induced contraction in the rabbit ear artery are supported mainly by release of intracellularly stored Ca^{2+} and influx of Ca^{2+} from the extracellular

medium, respectively (Bevan et al., 1973; Steinsland et al., 1973). A similar conclusion was reached by Urakawa and Holland (1964) from studies of transmembrane $^{45}\text{Ca}^{2+}$ fluxes in K^{+} -contracted guinea-pig tenia coli, although others have claimed differently (Imai and Takeda, 1967; Sunano and Miyazaki, 1968). For example, Sunano and Miyazaki (1968) reported that both the phasic and tonic component of the K^{+} contraction were initiated by inflow of extracellular Ca^{2+} in this tissue.

Recently, Hurwitz and co-workers (1980) suggested that two different types of voltage-sensitive Ca^{2+} channels are activated by K^{+} in isolated strips from the longitudinal layer of the guinea-pig ileum. They interpreted the two-component contractile response to K^{+} as reflecting a transmembrane inflow of extracellular Ca^{2+} through these separate Ca^{2+} channels. Thus, a release of Ca^{2+} from intracellular stores was not considered to be involved.

In the present investigation we have studied the influence of temperature and the electrolyte composition of the extracellular medium on the general characteristics and time course of K^{+} -evoked contractions in rat cerebral arteries. The effects of the Ca^{2+} -entry blockers nifedipine and lanthanum on the biphasic K^{+} response were also examined.

Materials and Methods

Vascular preparation. Adult Sprague-Dawley rats of either sex, weighing 200 to 300 g, were decapitated and the brain and medulla rapidly removed and placed in a chilled (8 - 12°C) modified NaKs (for composition, see below). The BA and the MCA were subsequently dissected free from the underlying brain tissue. The arteries were used immediately after excision or stored in the buffer (8 - 12°C) for no longer than 24 hr.

Measurement of mechanical activity. Ring segments of the arteries, 1 to 2 mm long, were suspended in a small volume muscle bath (2.5 ml), as previously described (Högestätt et al., 1983). Briefly, two small stainless-steel wires (diameter, 50 - 100 μ m) were gently inserted into the vessel lumen under microscope. Mechanical activity was recorded "isometrically" by means of a Grass Instruments FT03C force-displacement transducer. The signal from the transducer was displayed on a Grass Instruments model 7D polygraph. Over an equilibration period of about 60 min in Na⁺-Krebs buffer, the vascular preparation was stretched on repeated occasions so as to adjust the resting tension to approximately 2 mN. The temperature of the bath fluid was maintained at 37°C. A mixture of CO₂ (5 %) and O₂ (95 %) was continuously bubbled through the bicarbonate-based Krebs' solution in the muscle bath, resulting in a pH of approximately 7.4. The bath fluid was usually changed every 10 min and replaced by fresh buffer (also kept at 37°C and equilibrated with 5 % CO₂ and 95 % O₂). Tris-buffered solutions (for composition, see below) were bubbled with pure oxygen.

In the beginning of each experiment, the contractile capacity of the arterial segment was assayed by exposing the vessel to an isotonic K₂SO₄ (K₂S), containing 124 mM K⁺ (for composition, see below). This concentration of K⁺ was shown to induce an almost maximum contraction in the BA and MCA. Each preparation was repeatedly contracted by this solution (every 30 min) until at least two reproducible responses were obtained (a difference in maximum tension of 5 % or less between consecutive contractions was tolerated).

Temperature changes. The temperature of the bath solution was altered by switching over the thermoregulated water circuit, heating the muscle bath, to a separate pump system with the desired temperature. The arteries were always left to accommodate at the new temperature for 30 min before they were contracted by K^+ . The temperature was continuously registered by a small temperature probe placed in the organ bath.

Ca^{2+} depletion. Preparations were exposed to " Ca^{2+} -free" solutions for time periods varying between 0.5 min and 7 hr. During these periods the muscle bath was repeatedly rinsed with fresh Ca^{2+} -free buffer. K^+ and/or single concentrations of Ca^{2+} were introduced by completely changing the bath solution to a different one with the desired ion composition. This exchange was completed within 1 to 2 sec. Graded responses to Ca^{2+} in K^+ -depolarized vessels were obtained by adding small volumes of concentrated $CaCl_2$ solutions directly to the muscle bath.

$LaCl_3$ application. All experiments with $LaCl_3$ were performed on preparations bathing in carbonate- and phosphate-free, Tris-buffered solutions (with 1.5 mM $CaCl_2$) to avoid precipitation of lanthanum salts. $LaCl_3$ was added to the preparations 15 min before K^+ and the bath solution was replaced once (after 10 min) during this period.

Electrolyte solutions. The following buffers were used: 1) the modified NaKs was composed of (in millimolar): NaCl, 119; KCl, 4.6; $CaCl_2$, 1.5; $MgCl_2$, 1.2; $NaHCO_3$, 15; NaH_2PO_4 , 1.2; and glucose, 6.0; 2) the isotonic KKs was prepared by substituting all NaCl with an equimolar amount of KCl; 3) Tris-buffered solutions corresponding to the NaKs and the KKs were prepared according to the recipes above except for the following changes: the $NaHCO_3$ and the NaH_2PO_4 were replaced by 15 mM NaCl and 5 mM Tris (Tris(hydroxymethyl)aminomethane). The pH was titrated to 7.40 at 37°C by adding small volumes of concentrated HCl (6 N); 4) Ca^{2+} -free solutions always contained 0.01 mM EGTA, unless specified otherwise. Analytical grade chemicals and redistilled water were used for preparing all solutions.

Drugs. Verapamil hydrochloride (Knoll Pharmaceutical Company, Orange, NJ) and diltiazem hydrochloride (Tanabe, Tokyo, Japan)

were dissolved in water, and flunarizine dihydrochloride (Janssen Pharmaceutica, Beerse, Belgium) and felodipine (Hässle, Göteborg, Sweden) in ethanol. Nifedipine (Bayer, Leverkusen, FRG) was provided in ampoules, containing 0.1 mg/ml of nifedipine in 150 mg/ml of ethanol, 150 mg/ml of polyethylene glycol and water. The ampoules were kept in the dark until used to avoid light-induced degradation. The solvent used in the ampoules slightly increased the mechanical response to K^+ (< 5 %) but did not significantly change the tension in resting arteries. Dilutions were made in 0.9 % NaCl. All substances, including $LaCl_3$, were added directly to the organ bath in volumes of 5 to 25 μ l. The concentrations reported are the calculated final bath concentrations, assuming a complete dissociation of respective salt.

Statistics. Statistical analysis of the results was performed by using Student's t test for paired or unpaired data. The 0.05 level of probability was accepted as significant. The results shown in figures and the text are expressed as mean value $\pm$ S.E.M., where n denotes the number of arterial segments examined (all from different animals). Calculation of mean EC_{50} or IC_{50} values, i.e., the concentrations of the drugs producing half-maximum effect, was based on the geometrical mean. The individual concentration-response curves were drawn by hand and the EC_{50} or IC_{50} values determined graphically for each curve. Further details on materials and methods are given with the results.

Results

Response to K^+ . Exposure to the isotonic high K_Ks (containing 124 mM K^+) induced strong, stable and reproducible contractions in the rat MCA and BA. K^+ concentrations lower than 124 mM induced contractions smaller than that evoked by the K_Ks. Two distinct contraction components could be resolved, an early rapid phase and an ensuing slow phase, lasting throughout the K^+ exposure (fig. 1a). Immediately upon application of 124 mM K^+ , the tension rose rapidly and reached a first maximum after 9.4 ± 0.3 sec ($n=15$), thus forming the first component of contraction (phase A). After a brief relaxation, a slow sustained contraction developed (phase B). The amplitude of the K^+ response at the peak of the early transient contraction and 5 min after introduction of K^+ , i.e., at a time when the tension had usually stabilized, were used as measures of phase A and phase B, respectively. Phase A of the K^+ contraction was usually between 10 and 20 % smaller than phase B. When the K_Ks was withdrawn and replaced by Na_Ks, the arteries immediately relaxed. A biphasic contraction of the mentioned type was generally observed only after exposures to K^+ contractions higher than 30 mM.

Influence of $(Na^+)_e$ on the K^+ contraction. In order to evaluate the importance of the (Na^+) of the K_Ks for the mechanical response, the MCA and BA were exposed to K_Ks with Na^+ concentrations ranging between 0 and 135 mM. The ordinary K_Ks used to contract the vessels contained approximately 16 mM Na^+ . Replacement of this small amount of Na^+ by K^+ only slightly increased (≈ 5 %) the contraction. If the (Na^+) of the K_Ks was raised by adding various amounts of NaCl (0-119 mM) without simultaneously reducing the (KCl) (hypertonic solutions), the maximum of the response was gradually reduced. Although phase B of the contraction appeared to be somewhat more affected than phase A, the characteristic biphasic course of the response was still maintained (fig. 1b). However, an almost identical change of the contractile response was obtained by using a K_Ks made equally hypertonic by the addition of sucrose (fig. 1c). Exposure to a hypertonic Na_Ks containing 238 mM sucrose, evoked only a small, slowly developing contraction, amounting to 6.2 ± 1.2 % ($n=6$) of the maximum response to K^+ .

Effect of temperature on the K^+ contraction. Lowering of the temperature from 37°C to 30°C or 20°C was found to reversibly reduce the maximum of the contractile response to K^+ in the BA by $23 \pm 3\%$ ($n=6$) and $30 \pm 4\%$ ($n=8$), respectively (fig. 2). However, the slow component of the K^+ contraction tended to be slightly more affected by cooling than the early rapid phase. In contrast, when the temperature was increased from 37°C to 40°C , the tonic phase remained unchanged, whereas phase A was slightly suppressed. As the temperature was decreased, the two contraction components gradually became separated (fig. 2). It was also observed that the contractions developed faster at higher than at lower temperatures.

Effect of Ca^{2+} deprivation on the K^+ contraction. If Ca^{2+} was withdrawn from the muscle bath during the course of an established K^+ contraction by changing the bath solution to a different KKs with no added Ca^{2+} , the MCA and BA immediately relaxed. Exposure to a nominally Ca^{2+} -free KKs elicited a transient contraction. Even short preincubation periods in Ca^{2+} -free NaKs substantially reduced the transient response. The reduction of the two components of the K^+ contraction after various time periods in a nominally Ca^{2+} -free NaKs is shown in figure 3. As can be seen, Ca^{2+} deprivation considerably suppressed both components of the K^+ response and, after 5 min in "zero- Ca^{2+} " NaKs, phase A and phase B were reduced to approximately 10 and 2 % of their original size in normal Krebs' solution (1.5 mM Ca^{2+}).

In a separate series of experiments, the MCA and BA were preincubated in Ca^{2+} -deficient NaKs for 20 min before being exposed to the KKs (containing 1.5 mM Ca^{2+}). Notably, the KKs still evoked a biphasic contractile response under these conditions and the amplitudes of the individual contraction components were unaltered. However, the time to half-peak tension of phase A, was significantly increased ($P < .01$) from 1.64 ± 0.04 to 2.50 ± 0.12 sec ($n=6$). Even prolonged periods (3 - 7 hr) in Ca^{2+} -deprived medium (supplied with 1 mM EGTA), long enough to abolish or reduce the vasoconstrictor responses to serotonin (10 μM), NA (10 μM) (MCA only), prostaglandin $\text{F}_{2\alpha}$ (10 μM) and K^+ (124 mM) to a minimum, failed to suppress the amplitude of phase A. This was particularly apparent when the experiments were

performed at low temperature (20°C) (fig. 4a). Some arteries were exposed to a Ca^{2+} -deficient K⁺s during the last 10 min of the Ca^{2+} depletion period. This was considered to abolish active electrical responses by clamping the cell membrane at a depolarized state. Nonetheless, repletion of CaCl_2 (1.5 mM) to the depolarized arteries still elicited a phase A response (fig. 4b), although the intervening relaxation separating the two contraction components was considerably diminished and sometimes present only as a small inflexion on the rising part of the contraction curve.

Effect of La^{3+} on the K^+ contraction. All experiments with La^{3+} were performed in Tris buffer solution. Neither the maximum nor the biphasic pattern of the K^+ contraction were appreciably influenced by the choice of buffer. Normally, La^{3+} (0.1 μM - 1 mM) did not influence the tension of resting arteries. However, application of 10 mM La^{3+} occasionally induced a small transient contraction between 5 and 10 % of the maximum response to K^+ . In the BA, La^{3+} both inhibited K^+ -induced contractions and relaxed established contractures. After long exposures to K^+ (10 - 60 min) in the presence of La^{3+} (1 μM - 0.1 mM), the tonic component of the K^+ contraction usually "escaped" from inhibition (not shown). Due to this delayed tension increase, we used the maximum response obtained during the first 5 min of exposure to K^+ for quantifying the inhibition produced by La^{3+} . As shown in figure 5, a significant inhibition of the K^+ contraction was noticed already at a (La^{3+}) of 1 μM and a concentration of 1 mM La^{3+} completely abolished the response. Half-maximum inhibition was obtained at a (La^{3+}) of approximately 3 μM .

Effect of nifedipine on the K^+ contraction. Nifedipine, added to the bath solution 15 min before K^+ , preferentially suppressed the tonic component of the K^+ contraction, as compared to the initial rapid phase (fig. 6). Concentration-response curves for the inhibitory effect of nifedipine on phase A and phase B of the K^+ response in the BA are shown in figure 7. As can be seen, the nifedipine concentration required to obtain 50 % inhibition of phase A of the K^+ contraction was approximately 10 times higher than that needed to obtain the same degree of effect of phase B. The maximum inhibitions produced by nifedipine also differed

significantly between the two contraction components and were $57 \pm 4\%$ ($n=8$) for phase A and $84 \pm 2\%$ ($n=8$) for phase B ($P < .001$). A similar preference for the tonic component was displayed by diltiazem, verapamil, flunarizine, and felodipine, even though the potencies of these drugs differed markedly. Notably, the mechanical response to K^+ obtained in the presence of nifedipine concentrations higher than 10 nM could usually be resolved into three components (cf., Sunano, 1976): an early rapid phase (presumably corresponding to phase A), an ensuing small transient component, which reached a maximum approximately 30 sec after application of K^+ , and a final sustained contraction (corresponding to phase B) (see fig. 6).

As mentioned above, arteries pretreated in Ca^{2+} -deprived medium either in a polarized or in a K^+ -depolarized state still responded with a biphasic contraction when exposed to the Ca^{2+} -containing K_Ks. Interestingly, the inhibitory effect of nifedipine on phase A was considerably enhanced in these preparations, as compared with those which had been incubated with the drug in the continuous presence of extracellular Ca^{2+} . This is illustrated in figures 8 and 9. Segments of the BA were equilibrated with a preselected concentration of nifedipine (30 nM) either in the presence or in the absence of external Ca^{2+} . The exposure time for Ca^{2+} -free solution was 10 min. A second series of arteries were depolarized by K^+ during the last 5 min in Ca^{2+} -free medium (fig. 9). The amplitude of the contractions evoked by introducing the K_Ks were measured at three preset times: 1) approximately 10 sec after introduction of the K_Ks or at the maximum of the early rapid phase (when present), 2) 30 sec later or at the maximum of the second peak (when present), and 3) 5 min after the onset of the contraction (phase B). It is evident that these procedures considerably enhanced the inhibitory effect of nifedipine on the early rapid phase, as compared with the control situation. Prior depolarization of the muscle reduced also the second peak, leaving only a slow sustained contraction devoid of transient components. All these effects were reversible when Ca^{2+} was reintroduced.

The MCA and BA immersed in Ca^{2+} -free medium and depolarized by K^+ responded to Ca^{2+} (administered cumulatively) with concentration-related contractions. Identical graded responses

were produced in both types of artery. The threshold $(\text{Ca}^{2+})_e$ for contraction was $10\ \mu\text{M}$ and a maximum response was obtained at a $(\text{Ca}^{2+})_e$ of approximately $1\ \text{mM}$. The $(\text{Ca}^{2+})_e$ eliciting half-maximum effect was $64\ \mu\text{M}$ ($\log \text{EC}_{50} = -4.19 \pm 0.02$; $n=15$). If Ca^{2+} was added cumulatively at four repeated occasions to the same arterial segment with 30-min intervals, the responses to the lower concentrations of Ca^{2+} were gradually reduced, whereas the maximum response to Ca^{2+} remained fairly unchanged. Taken this spontaneous desensitization of the preparations into account, nifedipine given 15 min before Ca^{2+} still effectively inhibited the graded Ca^{2+} responses (fig. 10, left panel). In order to exclude that Ca^{2+} concentrations higher than $4\ \text{mM}$ might have overcome the nifedipine blockade in a competitive manner, the experiments were repeated in Tris solution. The $(\text{Ca}^{2+})_e$ could now be increased to $50\ \text{mM}$ without preprecipitation of calcium salts. The EC_{50} value for Ca^{2+} in Tris buffer ($\log \text{EC}_{50} = -4.17 \pm 0.02$; $n=11$) did not significantly differ from that obtained in Krebs' solution. In addition, Ca^{2+} concentrations higher than $10\ \text{mM}$ usually produced a gradual decay of the active tension. As can be seen in figure 10 (right panel), the inhibition produced by nifedipine could not be completely reversed even by increasing $(\text{Ca}^{2+})_e$ up to $50\ \text{mM}$.

Discussion

As shown in the present study, the contractile response to K^+ in isolated rat cerebral arteries consisted of two components, an early rapid phase and an ensuing slow phase, separated by a transient relaxation. The observation that the two components of the K^+ contraction could be clearly dissociated by cooling and separately influenced by various procedures suggests that the two contraction components are induced by different mechanisms. Although this pattern of response to K^+ is not seen in all muscular preparations, bimodal contractions of similar type have previously been demonstrated in a number of other smooth muscle tissues (for references, see Bolton, 1979). In most of these investigations, K^+ -rich solutions with a low Na^+ content were used in order to maintain physiological tonicity. Although Na^+ substitution has been reported to induce smooth muscle contraction (see, e.g., van Breemen et al., 1979), possibly by suppressing or reversing a Na^+ - Ca^{2+} exchange process, the contractions induced by the K_Ks in rat cerebral arteries appear not to be caused by the reduced $(Na^+)_{\text{e}}$, but rather by the raised $(K^+)_{\text{e}}$, for two main reasons. First, the contractions were only partly reduced by increasing the (Na^+) of the K_Ks from 16 to 135 mM. Secondly, a similar change of the contractile response was obtained by adding sucrose to the K_Ks instead of NaCl. Thus, the reduction of the response produced by increasing the NaCl content of the K_Ks seemed to reflect the hyperosmolarity of the solution rather than the specific concentration of Na^+ in the bath fluid.

Bevan et al. (1973) suggested that the bimodal response to exogenous NA in the rabbit ear artery was associated with two modes of excitation: an initial rapid myogenic propagation of excitation from the superficial cell layer(s) to the interior of the vessel wall, causing a fast transient contraction, and a subsequent diffusion of NA into the vessel wall, resulting in a slow (tonic) contraction. However, this does not seem to be the mechanism behind the biphasic contractile response to K^+ in rat cerebral arteries, as the vessel walls of the MCA and BA consist of only 2 to 3 layers of smooth muscle cells (Högestätt et al., 1983), and therefore any stimulant substance is expected to reach

the muscle cells almost simultaneously.

Electrophysiological studies have demonstrated that the initial fast component of the K^+ contraction in the guinea-pig tenia coli and in rat ileal smooth muscle is accompanied by membrane depolarization and the appearance of spike discharges (Shimo and Holland, 1966; Imai and Takeda, 1967; Syson and Huddart, 1973). The amplitude of the AP discharges decreased as the membrane depolarization advanced and finally ceased ("depolarization block") at approximately the same time as the initial phasic contraction merged into the tonic phase. From these observations it was suggested that the burst of spikes was the ultimate trigger of the phasic response to K^+ .

Because cerebrovascular smooth muscle from several species, including the rat, has been shown to possess the ability to generate active electrical responses (Harder, 1980; Karashima and Kuriyama, 1981; Hirst et al., 1982), a burst of spike potentials may be considered a feasible explanation also for phase A of the K^+ contraction in rat cerebral arteries. However, the finding that Ca^{2+} elicited an initial rapid response also in previously K^+ -depolarized arteries would discard such a mechanism. Similar observations were made by Hurwitz et al. (1980) on the guinea-pig tenia coli and by Sunano (1976) on the guinea-pig ureter. Johnishi and Sunano (1978) were even able to discriminate between the large phasic response evoked by K^+ depolarization in rat vas deferens and the small rapid contractions elicited by spike potentials, which occurred during the ascending part of the phasic response. Thus, the appearance of spike discharges and the accompanying large phasic response may not necessarily be causally related to each other. As electrophysiological recordings were not made in the present study, it cannot be excluded that AP discharges did occur during the early depolarization phase and, if so, that this might have contributed to the development of the early rapid phase of the K^+ contraction. Nonetheless, it seems quite clear from the present experiments that a phase A response can be elicited also in the complete absence of spike potentials.

K^+ may also, apart from directly stimulating the smooth muscle cells, in an indirect manner influence the contractile activity

by releasing transmitters from autonomic nerves. The transmitters may subsequently interact with receptors at the muscle cell membrane. Thus, the ultimate response to K^+ will be the result of a summation of direct and indirect effects. Such a neuronal transmitter release was shown to contribute to the transient relaxation observed in K^+ -contracted uterine strips from oophorectomized rats (Bengtsson, 1977). Propranolol and reserpine treatment greatly diminished the relaxation, indicating that beta-adrenoceptors were stimulated by endogenous NA released from adrenergic nerve endings. In contrast, propranolol (1 μM), phentolamine (1 μM), phenoxybenzamine (1 μM , 20-min exposure) and atropine (3 μM) failed to influence the course of the K^+ contraction in the rat BA (E.D. Högestätt, unpublished observations), indicating that adrenergic and cholinergic mechanisms are probably not involved in this response. In addition, the rat BA preparation seems to lack postjunctional alpha-adrenoceptors, as exogenous NA fails to elicit contraction (Högestätt et al., 1981; Hirst et al., 1982). Inasmuch as other types of autonomic nerve fibers have been suggested to be present in cerebral arteries, e.g., peptidergic nerves (Larsson et al., 1976, Edvinsson et al., 1981), a nervous component other than adrenergic or cholinergic superimposed on the direct action of K^+ on the VSM cells cannot be completely excluded.

It is generally agreed that the concentration of free intracellular Ca^{2+} is the main determinant of the degree of activation of the contractile apparatus in smooth muscle (Ebashi, 1980). Under steady-state conditions, those mechanisms which increase the myoplasmic (Ca^{2+}) are balanced by mechanisms which eliminate Ca^{2+} from the myoplasm, such as Ca^{2+} extrusion and intracellular Ca^{2+} binding and/or uptake. It is reasonable to assume that the change in tension which occurs during a contraction is brought about by an imbalance between these mechanisms. Theoretically, the brief relaxation observed during the early course of the K^+ contraction in the present experiments may be explained by activation of such an uptake or extrusion mechanism, which after a short period of time becomes either saturated by Ca^{2+} or deactivated. However, two pieces of evidence would speak against this suggestion. First, the transient relaxation was greatly

enhanced by cooling, which cannot be reconciled with an active energy consuming process. Secondly, the transient relaxation was present irrespective of the (Na^+) in the KKs. This would exclude the involvement of a Na^+ -dependent Ca^{2+} extrusion mechanism.

It is well established that the sources supplying activator calcium for the contractile process may change between consecutive phases of a single contraction (for references, see Bolton, 1979). In order to investigate the dependence of the two components of the K^+ contraction in rat cerebral arteries on inflow of extracellular Ca^{2+} , we used three experimental tools: 1) Ca^{2+} -free solutions, 2) nifedipine, and 3) LaCl_3 . Nifedipine and other so called "organic Ca^{2+} -entry blockers" are generally considered to produce VSM relaxation by reducing the entry of Ca^{2+} into the smooth muscle cells (Fleckenstein, 1977; Andersson and Högestätt, 1984). More specifically, drugs like nifedipine have been suggested to selectively reduce the passage of Ca^{2+} through potential-operated Ca^{2+} channels in the smooth muscle membrane (Bolton, 1979), in analogy with its selective blocking effect on the "slow channel" in heart muscle (Fleckenstein, 1977; Kohlhardt and Fleckenstein, 1977; Nayler and Poole-Wilson, 1981). However, others have advocated that Ca^{2+} -entry blockers also may interfere with receptor-operated Ca^{2+} channels in certain VSM preparations (Towart, 1981; Cauvin et al., 1982). Intracellular sites of action of these drugs, rather than inhibition of transmembrane Ca^{2+} transport, have also been suggested (Church and Zsotér, 1980; Boström et al., 1981). Presently, Ca^{2+} entry blockade still seems to be the main mechanism behind the smooth muscle relaxant effect of nifedipine, even if other effects may contribute (Andersson and Högetätt, 1984). The three valent La ion, on the other hand, is believed to function as a relatively nonselective inhibitor of transmembrane Ca^{2+} transport by occupying negatively charged membrane sites involved in Ca^{2+} translocation (van Breemen et al., 1972; Weiss, 1974).

Hurwitz and McGuffee (1978) suggested that Ca^{2+} , before inward displacement, reversibly interacts with saturable transport sites in the smooth muscle membrane. Theoretically, nifedipine may reduce Ca^{2+} entry simply by competing with Ca^{2+} for these sites. This view is supported by experiments showing that the

effect of nifedipine can be reversed by increasing $(Ca^{2+})_e$ (Rosenberger et al., 1979; Hay and Wadsworth, 1982; Spedding, 1982). Even if these studies would seem to implicate competitive antagonism, the experimental results are also compatible with nifedipine acting at a separate site on the Ca^{2+} channels different from the Ca^{2+} binding site. In K^+ -depolarized rat cerebral arteries, nifedipine inhibited Ca^{2+} -induced graded contractions in an apparently noncompetitive fashion (present study). However, the results are somewhat difficult to interpret, as high concentration of Ca^{2+} usually decreased the active tension.

As shown in the present study, the tonic component of the K^+ contraction was critically dependent on the presence of Ca^{2+} in the bath medium. Reintroduction of Ca^{2+} to arteries previously depolarized by K^+ in the absence of external Ca^{2+} immediately restored the contraction. Although phase A of the K^+ contraction appeared to be more resistant to Ca^{2+} withdrawal than phase B, its maximum rapidly decayed with time in Ca^{2+} -free medium and after 5 min the response was reduced to approximately 10 % of its original size. For comparison, the maximum response to serotonin (10 μ M) was reduced only by approximately 55 % after the same treatment (E.D. Högestätt, unpublished observation). The observations that short periods in Ca^{2+} -free medium considerably reduced the biphasic K^+ contraction and that a relatively low concentration of La^{3+} (1 mM) abolished the response imply that extracellular Ca^{2+} is necessary to initiate both components of the K^+ contraction. A similar conclusion was reached by Sunano and Miyazaki (1968), Hurwitz et al. (1980), and Shimodan and Sunano (1981) for the K^+ contraction in guinea-pig vas deferens and ileal smooth muscle. The small phasic contractile response to K^+ , which persisted for a few min in Ca^{2+} -deficient solution (present study), does not necessarily suggest intracellular Ca^{2+} release, but may rather reflect influx of Ca^{2+} derived from a less readily depleted membrane associated Ca^{2+} pool.

The Ca^{2+} entering the muscle cells after K^+ stimulation may initiate contraction in two possible ways. First, Ca^{2+} may pass through the membrane into the cytoplasm and by itself directly activate the contractile apparatus. From measurements of trans-

membrane $^{45}\text{Ca}^{2+}$ fluxes using the La^{3+} method, van Breemen et al. (1972) concluded that the K^{+} -induced Ca^{2+} uptake into the VSM cells of the rat aorta was sufficiently large to account for the contractile response. Secondly, the Ca^{2+} entering the cells may trigger release of intracellularly stored Ca^{2+} . Such a mechanism of Ca^{2+} -induced Ca^{2+} release has been suggested to occur in the heart and skeletal muscle (Endo et al., 1970; Fabiato and Fabiato, 1975). However, prolonged treatment of the rat MCA and BA in Ca^{2+} -free, EGTA-containing solution, which abolished or reduced the contractile responses to serotonin, NA (MCA only), prostaglandin $\text{F}_{2\alpha}$ and K^{+} to a minimum and thus is expected to have depleted all intracellular stores of activator calcium, were shown to reduce neither phase A nor phase B of the contraction induced by the simultaneous addition of K^{+} and Ca^{2+} . This does not support the view that the early rapid component is due to release of intracellular Ca^{2+} , nor does it favor a trigger function for Ca^{2+} in K^{+} -activated rat cerebral arteries. Thus, all Ca^{2+} used for activating the contractile process after K^{+} stimulation appears to originate from the extracellular medium.

Nifedipine preferentially inhibited phase B of the K^{+} contraction and a 10-fold higher concentration of the drug was usually required to obtain the same degree of effect on phase A. The Ca^{2+} -entry blockers verapamil, diltiazem, flunarizine and felodipine also produced a larger inhibition of the tonic component than of the early rapid component of the K^{+} response. This suggests that Ca^{2+} entry into the VSM cells during the two contraction components occurred via different pathways. Hurwitz et al. (1980) recently proposed that the biphasic response to K^{+} depolarization in the longitudinal muscle of the guinea-pig ileum reflects the operation of two separate groups of K^{+} -activated Ca^{2+} channel mechanisms with different kinetic properties. This may be valid also for the K^{+} contraction in rat cerebral arteries. The occurrence of two separate K^{+} -activated Ca^{2+} channels in the cell membrane, one permitting a rapid and transient movement of Ca^{2+} across the membrane (the "rapidly activated Ca^{2+} channel"), the other allowing Ca^{2+} to pass at a slower rate (the "slowly activated Ca^{2+} channel"), can satisfactorily explain the present experimental results. Although these channels are referred to as Ca^{2+} channels, it cannot be excluded that other ions, e.g., Na^{+} ,

may also traverse the channels.

The brief duration of the initial rapid component of the K^+ response, as opposed to the slow component, suggests that the rapidly activated Ca^{2+} channel is inactivated shortly after the onset of the K^+ contraction. Membrane depolarization alone was not sufficient to cause inactivation of the channel, as reintroduction of Ca^{2+} to arteries previously depolarized by K^+ in Ca^{2+} -free medium still produced a phase A response. Rather, the inactivation of the channel may be mediated by Ca^{2+} accumulating at the cytoplasmic side of the plasmalemma as a result of Ca^{2+} entry or by Ca^{2+} binding to a regulatory site within the pore itself. Ca^{2+} -dependent regulation of ion conductances has previously been implicated for both Ca^{2+} and K^+ channels in a variety of tissues (Eckert et al., 1981; Hagiwara and Byerly, 1981).

The relatively greater sensitivity of the tonic component of the K^+ contraction to the Ca^{2+} -entry blocker nifedipine, as compared with the early fast component, would indicate that the drug preferentially blocked the slowly activated Ca^{2+} channel. However, if the arteries were incubated with nifedipine in the absence of extracellular Ca^{2+} , the inhibition of phase A was considerably enhanced. This effect was even more pronounced if the vessels were also depolarized by K^+ during the incubation period. Thus, the presence of Ca^{2+} in the bath fluid somehow exerts a protective effect against the inhibitory action of nifedipine. This might indicate that nifedipine, in the absence of extracellular Ca^{2+} , can occupy binding sites associated with the rapidly activated Ca^{2+} channel which are inaccessible to the drug in Ca^{2+} -containing medium.

Altogether, the results of the present study suggest that high K^+ solutions stimulate contraction in rat cerebral arteries by the opening of two separate types of Ca^{2+} channel, one permitting a rapid and transient movement of Ca^{2+} across the smooth muscle membrane (the rapidly activated Ca^{2+} channel), the other allowing Ca^{2+} to pass at a slower rate (the slowly activated Ca^{2+} channel). This suggestion is not in conflict with the general view that high K^+ solutions induce VSM contraction mainly by depolarization

of the plasma membrane, leading to activation of potential-operated Ca^{2+} channels and subsequent Ca^{2+} entry (Bolton, 1979). It may rather imply that potential-operated Ca^{2+} channels in VSM represent a heterogeneous population, which may be further subdivided.

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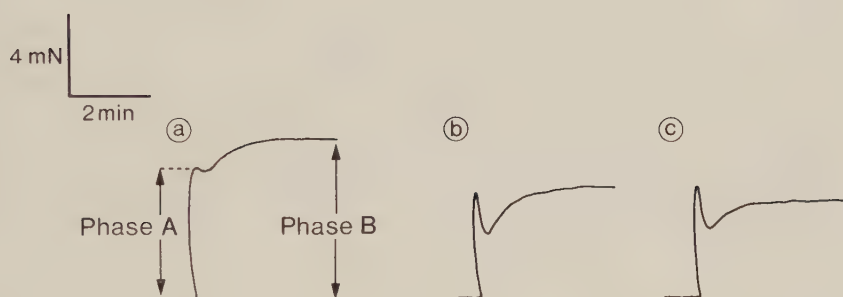


Fig. 1. Contractions induced by K^+ -rich solutions with different compositions in an isolated segment of the rat BA. **a**, biphasic contractile response induced by an isotonic high K^+ (124 mM), low Na^+ (16 mM) solution. The two phases of the contraction are indicated on the graph. **b**, mechanical response to a hypertonic K^+ (124 mM)-rich solution, prepared by adding KCl to a NaKs without concomitantly reducing the (NaCl). **c**, contraction induced by a high K^+ (124 mM), low Na^+ (16 mM) solution made equally hypertonic as the solution in (b) by the addition of 238 mM sucrose.

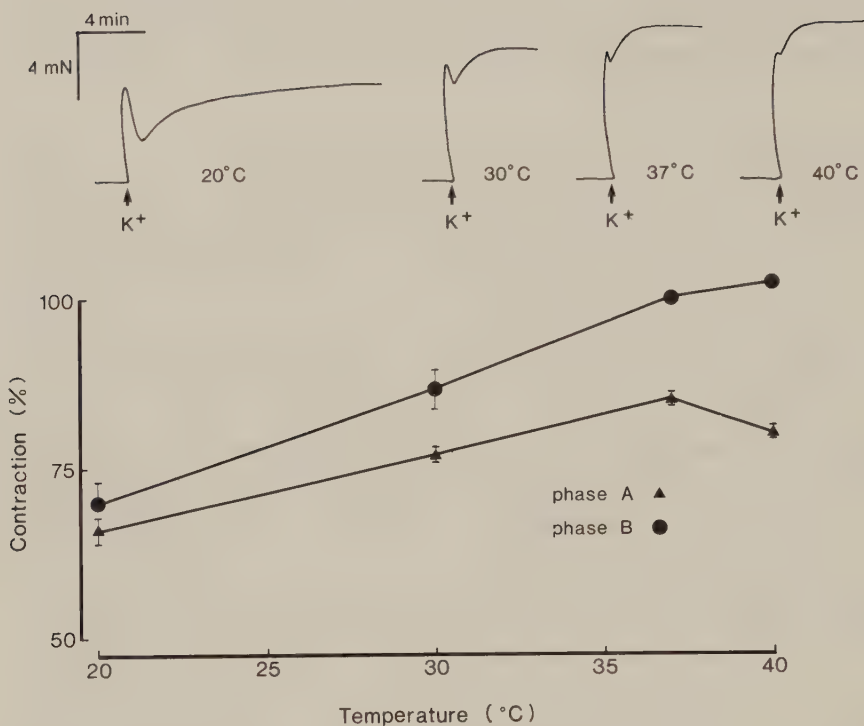


Fig.2. Influence of temperature on the biphasic mechanical response to 124 mM K⁺ in the isolated rat BA. Each temperature change was preceded by two control contractions at 37°C. The mean value of phase B of these two contractions was taken as 100 %. Because the contractions developed slower at low temperatures (e.g., at 20°C of the slow phase required 10-20 min to be fully developed), the amplitude of phase B was measured at a point where the contractions had stabilized, n=6-8. Tracings show the time course of the K⁺ contraction at the various temperatures, as recorded from a single arterial segment. For further details, see "Materials and Methods".

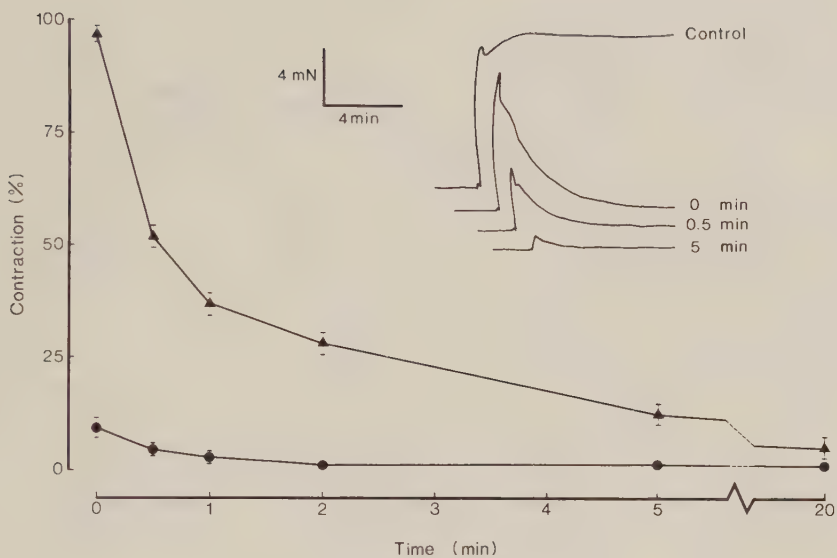


Fig. 3. Reduction of the two components of the K^+ contraction in the isolated rat BA after various time periods (varied randomly) in a nominally Ca^{2+} -free Krebs solution. A Ca^{2+} -free, high K^+ (124 mM) solution was used to initiate contraction (except for the controls). The arterial segments were allowed to recover in NaKs (1.5 mM Ca^{2+}) for 20 min after each exposure to K^+ . The amplitude of each contraction component in Ca^{2+} -containing medium was set to 100 %. ▲, phase A. ●, phase B. The abscissa denotes the pre-incubation time in Ca^{2+} -deprived medium before addition of K^+ . Note the discontinuity on the time scale, $n=6$. Inset: superimposed tension tracings from a single vessel segment. For further details, see "Materials and Methods".

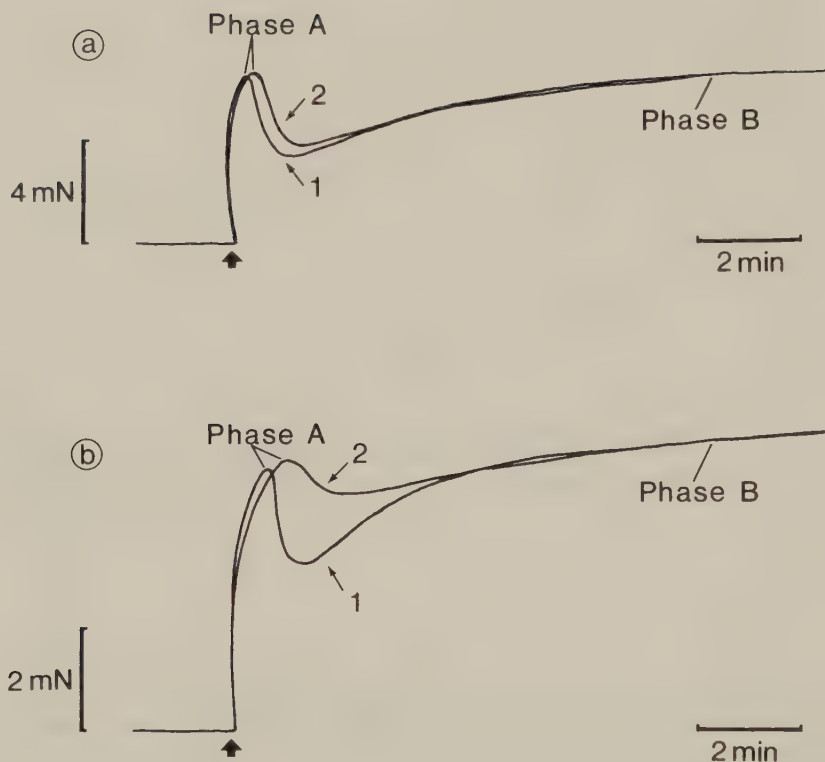


Fig. 4. **a**, superimposed tracings of contractions in a rat BA evoked 1) by 124 mM K^+ in normal Krebs solution (1.5 mM Ca^{2+}) and 2) by the simultaneous addition of K^+ and 1.5 mM Ca^{2+} after a 3-hr pretreatment in Ca^{2+} -deprived medium, containing 1 mM EGTA. During this period, the preparation was repeatedly exposed to K^+ (124 mM), serotonin (10 μ M), and prostaglandin $F_{2\alpha}$ (10 μ M) until the vasoconstrictor responses to these stimulants were abolished or reduced to a minimum. **b**, superimposed tracings of contractions in a rat BA induced 1) by 124 mM K^+ in normal Krebs solution (1.5 mM Ca^{2+}) and 2) by 1.5 mM Ca^{2+} in a previously K^+ -depolarized artery depleted of Ca^{2+} as described in **a**. The experiments were performed at 20°C. Arrows indicate when the bath medium was changed to the contracting solution.

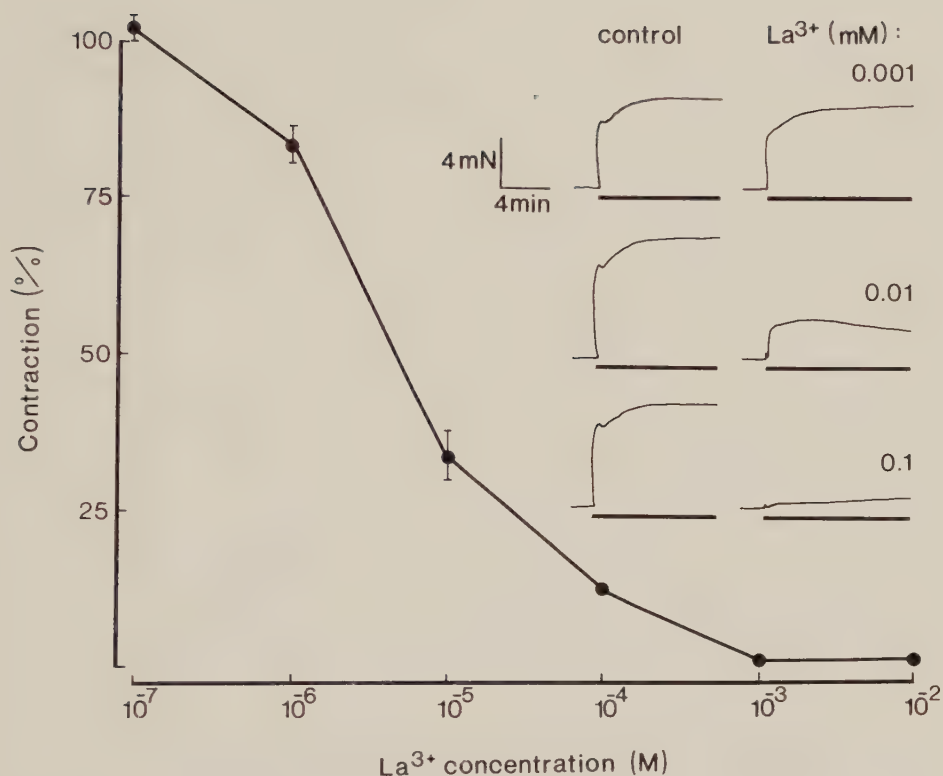


Fig. 5. Inhibition by La^{3+} of the contractile response to 124 mM K^+ in the isolated rat BA. The experiments were performed in Tris-buffered solutions. The maximum response obtained during the first 5 min of exposure to K^+ was used for quantifying the inhibition produced by La^{3+} ; 100 % on the ordinate denotes the response to K^+ before addition of LaCl_3 . The exposure time for LaCl_3 was 15 min. Each arterial segment was subjected to only one concentration of LaCl_3 , $n=5$. Inset: time course of the K^+ contraction before and after application of LaCl_3 . Horizontal bars indicate the presence of high K^+ medium. For further details, see "Materials and Methods".

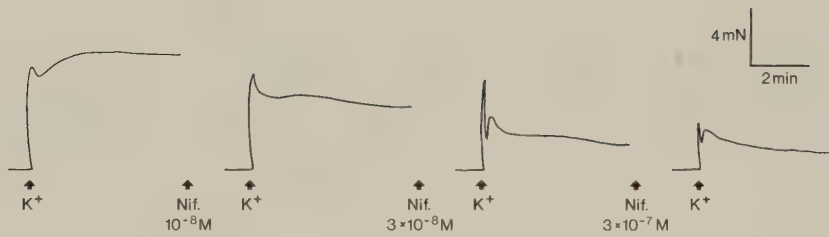


Fig. 6. Effect of increasing concentrations of nifedipine (Nif) on contractions evoked by 124 mM K⁺ in an isolated segment of the rat BA. The drug was present in the bath solution 15 min before introduction of K⁺ and during the contraction. Note the time course of the contraction curve and the appearance of "triphasic" K⁺ response at high Nif concentrations (cf., Sunano, 1976).

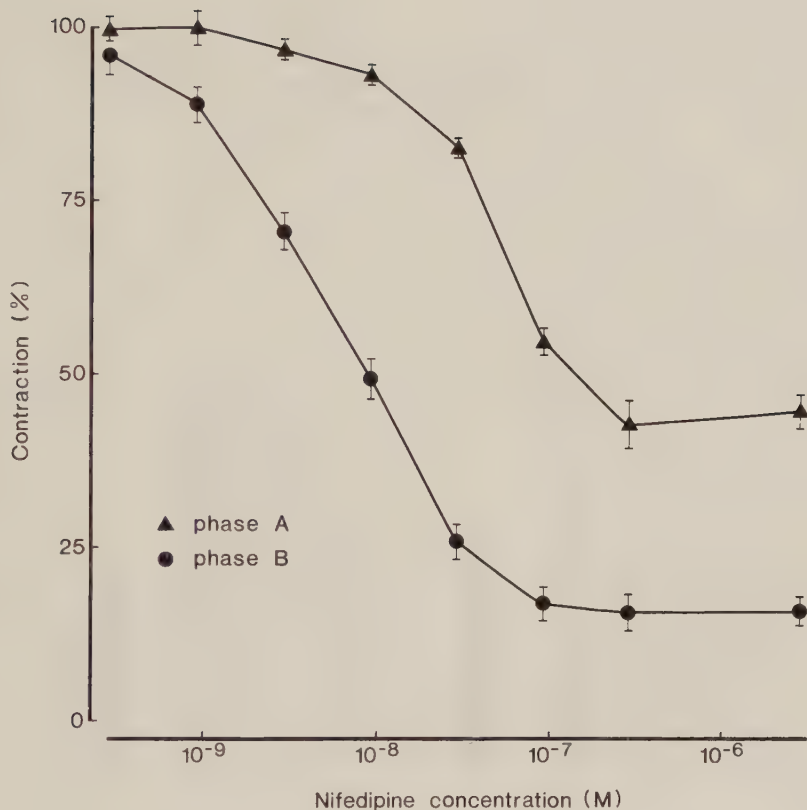


Fig. 7. Inhibition of the early rapid ($\blacktriangle$) and the slow ($\bullet$) phase of the K^+ (124 mM)-induced contraction by increasing concentrations of nifedipine in the rat BA. The exposure time for nifedipine was 15 min. Each vessel segment was subjected to three consecutive concentrations of the drug. Responses are expressed as a percentage of the corresponding fast and slow component of the K^+ contraction before addition of nifedipine, $n=6-8$.

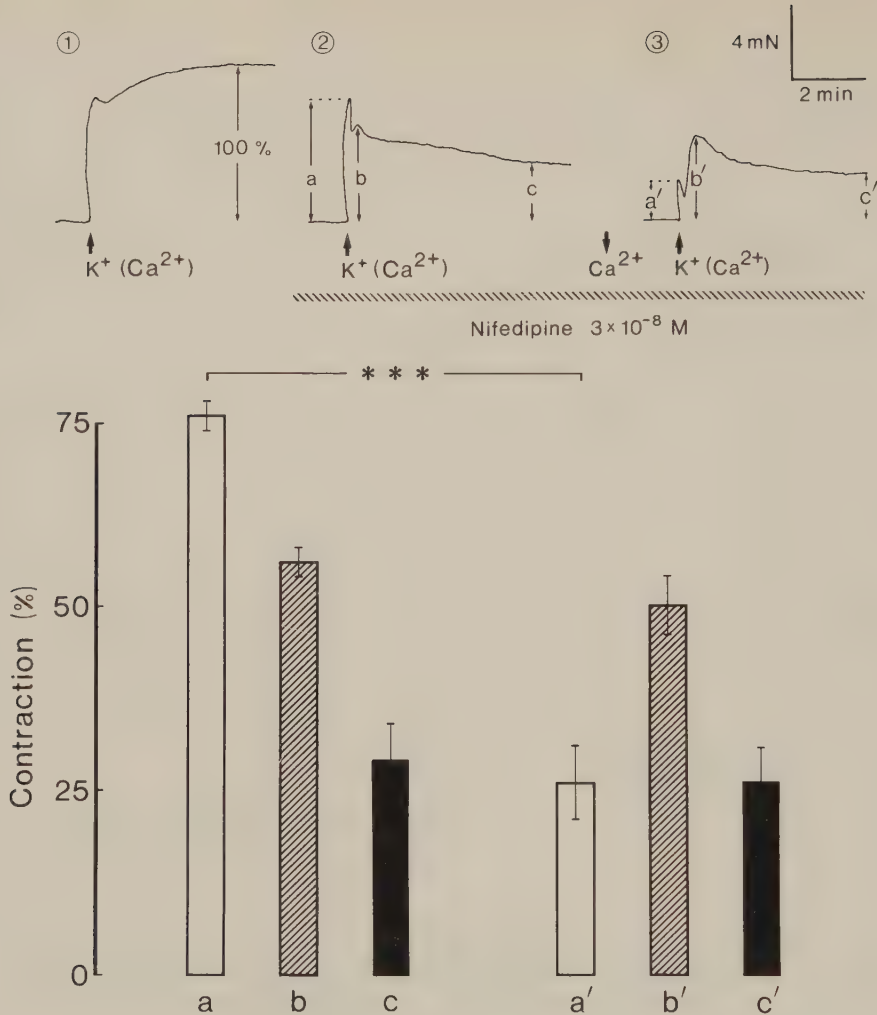


Fig. 8. Inhibitory effect of nifedipine on K^+ -induced contractions in the isolated rat BA. The preparations were incubated with the drug either in the presence or absence of external Ca^{2+} . All contractions were evoked by a 124 mM K^+ solution, containing 1.5 mM Ca^{2+} . Nifedipine was given 15 min before K^+ stimulation and kept present during the contractions. The experimental protocol used is described in the upper panel. 1) control contraction evoked by K^+ in normal Krebs solution; 2) response to K^+ after inhibition by 30 nM nifedipine; and 3) contraction evoked by the simultaneous addition of K^+ and Ca^{2+} after a 10-min preincubation period in Ca^{2+} -deprived medium with nifedipine. The amplitude of the K^+ contraction in the presence of 30 nM nifedipine was measured at three preset times, indicated by a (a'), b (b'), and c (c'), and expressed as a percentage of the control response to K^+ in normal Krebs solution (1). *** $p < 0.001$. For further explanations, see "Results".

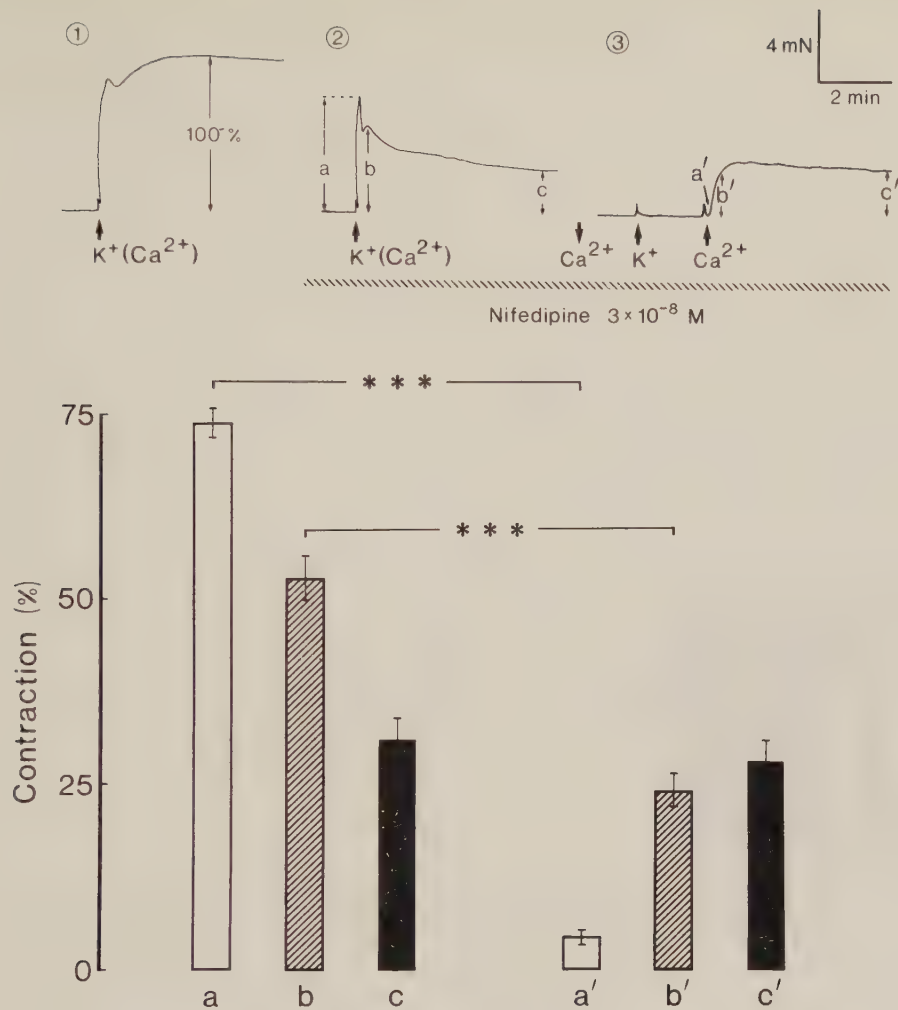


Fig. 9. Comparison between the inhibitory effects of nifedipine on K^+ - and Ca^{2+} -induced contractions in the isolated rat BA. The experimental protocol used is shown in the upper panel. 1) control response to K^+ in normal Krebs solution; 2) response to K^+ in the presence of 30 nM nifedipine; and 3) contraction induced by 1.5 mM Ca^{2+} in previously K^+ -depolarized muscle exposed to nifedipine. The Ca^{2+} -induced contraction was preceded by a 10-min preincubation period in Ca^{2+} -free medium. The preparation was depolarized by 124 mM K^+ during the last 5 min of this period. For further explanations, see legend to figure 8.

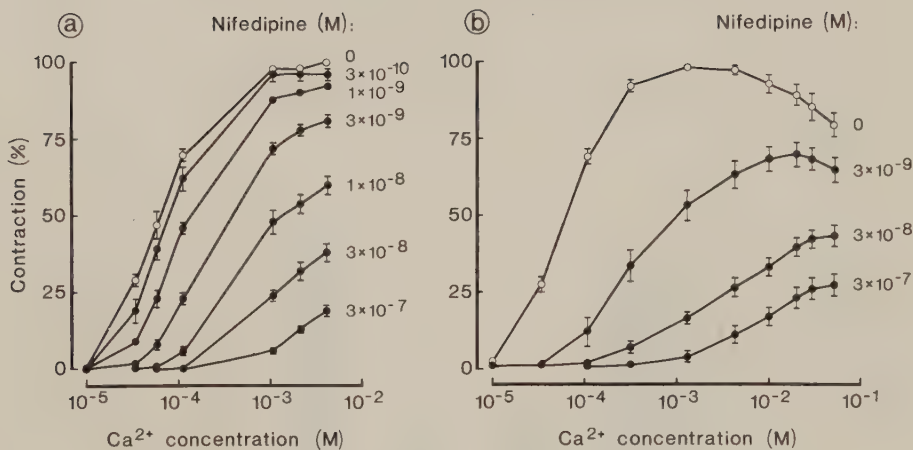
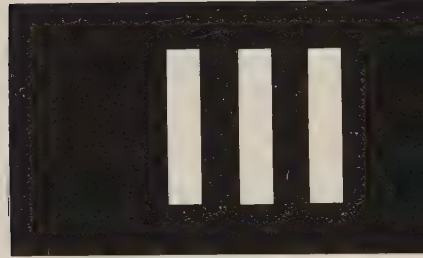


Fig. 10. Effect of nifedipine on the concentration-response curve for Ca^{2+} in K^+ (124 mM)-depolarized rat BAS. All preparations were treated in Ca^{2+} -free medium for 20 min and then exposed to a K^+ -rich solution, also lacking Ca^{2+} , for an additional 10-min period before each cumulative addition of Ca^{2+} . The exposure time for nifedipine was 15 min; 100 % of the ordinate denotes the maximum response to Ca^{2+} in the absence of nifedipine. Left panel: experiments performed in Krebs solution. Each vessel segment was exposed to three consecutive concentrations of nifedipine, $n=6-8$. The controls ($n=14$) were pooled together. Right panel: experiments performed in Tris buffer. Each arterial segment was exposed to only one or two concentrations of nifedipine, $n=4-5$. The controls ($n=9$) were pooled together.



Effects of nifedipine on potassium-induced contraction and noradrenaline release in cerebral and extracranial arteries from rabbit

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The present study was designed to evaluate the effects of the calcium antagonist nifedipine on potassium-evoked contractions and release of noradrenaline from sympathetic nerves in rabbit basilar and facial arteries. Contractions were measured isometrically in a small volume organ bath. While noradrenaline (NA) produced strong contractions in facial arteries, the majority of the basilar arteries responded only to the highest concentrations of NA employed ($>10 \mu\text{M}$) with weak contractions. Prazosin ($1 \mu\text{M}$) and phentolamine ($1\text{--}10 \mu\text{M}$) effectively antagonized the responses to NA in both types of vessel. In contrast, contractions evoked by potassium (K^+ , 124 mM) were only slightly reduced by the alpha-adrenoceptor blocking agents, indicating that the participation of endogenous NA in maintaining the contractile response to K^+ is either small or negligible in the vessel types studied. Nifedipine concentration-dependently inhibited K^+ -induced contractions in basilar and facial arteries, the former being significantly more affected as evidenced by the maximum inhibitions ($\sim 80\%$ compared to $\sim 60\%$) and IC_{50} values ($\sim 10 \text{ nM}$ vs. $\sim 30 \text{ nM}$). A combination of nifedipine ($0.3 \mu\text{M}$) and prazosin ($1 \mu\text{M}$) or phentolamine ($1 \mu\text{M}$) further suppressed the K^+ -evoked contractile response in facial arteries, but failed to do so in basilar arteries, when compared with the effect of nifedipine alone. The depressant effect of the alpha-adrenoceptor blockers was, however, still obtainable after reserpine treatment of the facial artery *in vitro*. Fluorescence histochemical demonstration of noradrenaline revealed a dense network of adrenergic nerve fibres in the walls of the basilar and facial artery. The vessels were also shown to accumulate ^3H -NA and release it upon depolarization with K^+ . The uptake and subsequent release of ^3H -NA were significantly reduced by desipramine ($10 \mu\text{M}$). Nifedipine ($0.3\text{--}3.0 \mu\text{M}$) failed to alter the K^+ -evoked ^3H -NA efflux from sympathetic nerves in neither of the two vessel types. It may be concluded that nifedipine effectively inhibits K^+ -evoked contractions in isolated basilar and facial arteries from rabbit without interfering with nerve-mediated NA release. Possible explanations for this selective effect of nifedipine on muscle contraction are discussed.

Key words: Ca^{++} antagonism, contraction, noradrenaline release, potassium, nifedipine

It is generally believed that calcium is intimately involved in the coupling between excitation and contraction in smooth muscle (Somlyo & Somlyo 1968*a*, Bolton 1979) and between stimulation and secretion in secretory tissues (Rubin 1970, Kirpekar 1975). Cytoplasmic free calcium (Ca^{++}) is considered to trigger the activation of the contractile proteins in muscle (Ebashi 1980) and release of transmitter from nerve endings (Miledi 1973). A number of regulatory systems controlling the Ca^{++} level have been suggested to operate in the smooth

muscle cell, among these translocation and uptake of Ca^{++} from intracellular storage sites (e.g. plasma membrane, sarcoplasmic reticulum and mitochondria; Somlyo & Somlyo 1968*b*, 1976, Somlyo et al. 1971, van Breemen et al. 1980), specific membrane channels permitting entry of extracellular Ca^{++} to the cell interior (Bolton 1979) and an ATP-dependent pump mechanism extruding Ca^{++} from the cytoplasm (Casteels 1980). Similar mechanisms have been proposed to operate in nerve terminals (Alnaes & Rahamimoff 1975, Blaustein et al. 1978,

Brinley et al. 1977, Henkart 1980). However, the functional importance of the different Ca^{++} pools in supplying activator Ca^{++} for contraction of smooth muscle and for release of transmitter substances from nerves has not been definitely established.

The quantity of noradrenaline (NA) released from sympathetic nerves upon depolarization of the pre-synaptic membranes depends on the concentration of extracellular Ca^{++} (Boullin 1967, Kirpekar & Wakade 1968). Depletion of Ca^{++} from the buffer medium attenuates or abolishes transmitter release induced by potassium (Kirpekar & Wakade 1968) as well as by electrical field stimulation (Boullin 1967), but not by tyramine (Lindmar et al. 1967). Contraction of vascular smooth muscle shows a similar sensitivity to changes in the extracellular Ca^{++} concentration (Hudgins & Weiss 1968, Lederballe Pedersen et al. 1978, van Breemen & Siegel 1980).

Recently, a group of drugs referred to as Ca^{++} antagonists (Fleckenstein 1977) have been utilized as pharmacological tools whereby specific mechanisms involved in Ca^{++} -requiring processes may be elucidated and characterized. These drugs have been suggested to interfere with potential sensitive Ca^{++} channels in the smooth muscle membrane and thereby reducing the passage of Ca^{++} to the intracellular compartment, resulting in a relaxation (Fleckenstein 1977, Bolton 1979).

A greater susceptibility of isolated cerebral arteries to these drugs than of peripheral arteries has been demonstrated (Hayashi & Toda 1977, Allen & Banghart 1979, Shimizu et al. 1980, Towert & Kazda 1980), suggesting that the contractile activity in cerebral vessels is more dependent on influx of extracellular Ca^{++} than that in peripheral vessels.

Although Ca^{++} antagonists have consistently been shown to depress vascular muscle contractility (Schümann et al. 1975, Fleckenstein 1977, Mikelsen et al. 1979, Shimizu et al. 1980), discrepancies exist concerning their effects on release of noradrenaline from sympathetic nerves (Haeusler 1972, Starke & Schümann 1973, Göthert et al. 1979). The present study was therefore undertaken to investigate the effects of the Ca^{++} antagonist nifedipine on potassium-induced contraction and noradrenaline release from adrenergic nerves in intra- and extracranial arteries from rabbit. The contribution of released noradrenaline to the development and maintenance of tension induced by high potassium solutions in isolated vessels from the two vascular regions was also examined.

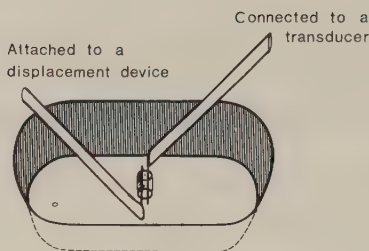


Fig. 1. Schematic drawing of the organ bath. Two steel wires (diameter = 100 μm) have been inserted into the vessel lumen. One metal prong is connected to a Grass FT03C force displacement-transducer, whereas the other is attached to a displacement device for adjusting the resting tension.

MATERIALS AND METHODS

Preparation

Adult rabbits of either sex, weighing approximately 2–3 kg, were sacrificed by injecting air (~20 ml) into a major auricular vein. The skull was opened, the brain rapidly removed and immersed into a chilled (+12°C) Na-Krebs solution (for composition see below), and the basilar artery dissected free from the underlying brain tissue. The facial arteries were surgically exposed bilaterally and excised from their origin at the common carotid artery to their first bifurcation. The outer diameters (in situ) of the basilar and facial arteries were approximately 300 μm .

The entire basilar artery and a 15 mm long segment of the facial artery were cleaned from surrounding tissues and either divided into 3 mm long segments for recording of mechanical activity, or cut open longitudinally and used for studies of transmitter release or used for fluorescence histochemistry. The vessels were used immediately after sacrifice of the animals unless otherwise stated.

Recording of mechanical activity

A system of L-shaped metal holders, placed in a temperature-controlled organ bath, was used for recording of isometric contractions. The organ bath contained 2.5 ml Krebs buffer, kept at $37 \pm 0.5^\circ\text{C}$. The buffer was continuously aerated with 5% CO_2 and 95% O_2 , maintaining the pH at approximately 7.4 (frequently checked by a Radiometer PHM 82 standard pH meter). Under microscope two small steel wires ($\varnothing = 100 \mu\text{m}$) were inserted into the vessel lumen (see Fig. 1). Wall tension was recorded isometrically by means of a Grass FT03C force-displacement transducer connected to one of the two metal prongs, whereas the other was attached to a displacement unit, allowing precise adjustments of the resting tension. The output from the transducer was amplified by and displayed on a Grass model 7 polygraph. The resting tension was slowly increased in a stepwise manner, each time by 0.5 mN, and the artery allowed to relax inbetween the extensions. During an equilibration period of 1 h, the passive force was adjusted to approximately 4 mN. As reproducible re-

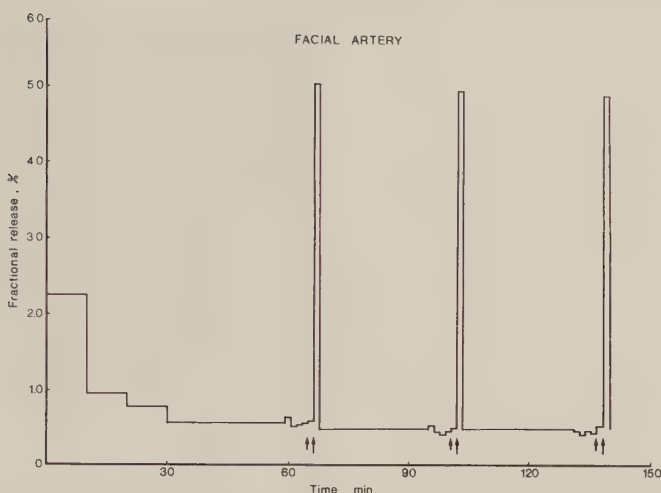


Fig. 2. The effect of K^+ exposure on the fractional release of 3H -NA from a rabbit facial artery. The tritium efflux was calculated as a fraction (in %) of the total tissue radioactivity released per min. Short and long arrows indicate when 40 mM and 124 mM K^+ were introduced, respectively.

sponses were obtained at this resting tension (see Results), it was maintained throughout the experiment. The bath fluid was changed every 15 min.

Normalization of contractile responses

Each artery was repeatedly contracted by a potassium (K^+) rich solution (124 mM; for composition see below) every 30 min until at least two reproducible contractions were obtained (the maximum tensions changed by less than approximately 5%). All subsequent contractions were expressed as a percentage of the mean of these. The reasons for using K^+ contractions as reference were manifold: The responses were stable, reproducible and appeared to provide a good approximation of the amount of smooth muscle present in the vessel walls, since an almost maximum activation of the contractile machinery was achieved and arteries of equal size elicited contractions of similar amplitude. The maximum response was always used as an estimate of the contractile activity, regardless of whether the contraction was transient or stable and remained at the maximum level. The different concentrations of nifedipine were always added to the organ bath 15 min prior to a subsequent K^+ exposure.

Fluorescence histochemistry

Catecholamines were demonstrated by the Falck-Hillarp histofluorescence method (Björklund et al. 1972). Pieces of arteries taken from the different regions were cut open longitudinally, stretched flat on microscope slides and dried by the hot stream from a hair dryer for 5 min. They were then transferred to an exsiccator and vacuum dried

over phosphorous pentoxide for at least 1 h. The vessel segments were subsequently exposed to paraformaldehyde vapour at 80°C for 1 h, mounted in Entellan (Merck) and examined in a Leitz Dialux 20 fluorescence microscope.

Transmitter release

Silk ligatures were tied to both ends of the arterial preparation, which was slightly stretched, and attached to the outside of a capillary glass tube. To facilitate rinsing of the preparation and to avoid trapping of radioactive transmitter within the vessel lumen, the arteries were cut open longitudinally which allowed the buffer also to circulate at the intimal side of the vessel. The capillary tube with attached vessel was transferred through a series of test tubes, each supplied with 3 ml buffer solution and placed in a water bath kept at $37 \pm 0.5^\circ C$. A stream of 5% CO_2 and 95% O_2 was conveyed through the capillary tube, giving a pH of approximately 7.4. The preparation was left to accommodate for 1 h in the first test tube containing normal Krebs buffer. Labelling of noradrenaline stores was achieved by incubating the preparation in a solution containing $0.23 \mu M$ tritiated *d, l*-noradrenaline (3H -NA; New England Nuclear, 15 mCi/ml) for 1 h. At the end of the incubation period the arterial segments were quickly rinsed three times in fresh and pre-warmed Krebs solution to diminish extracellularly bound 3H -NA. Monitoring of the radioactivity had in a preliminary test series revealed that the tritium efflux from the vascular segments reached a steady state 30 min after the end of the incubation period, and the experimental protocol was designed accordingly (Fig. 2).

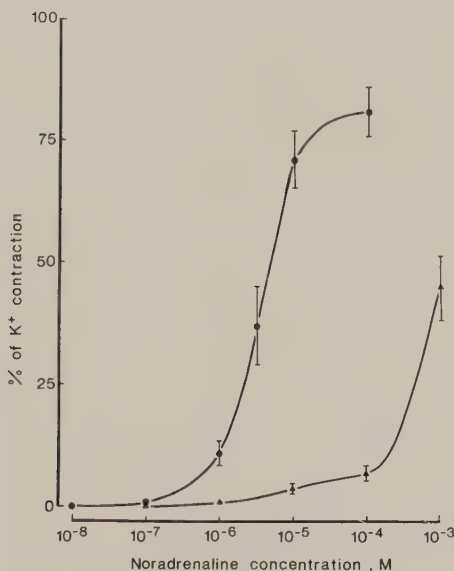


Fig. 3. Concentration-response curves for NA in rabbit facial (●) and basilar (▲) arteries. The responses are expressed as a percentage of the mean of two K⁺-induced (124 mM) contractions obtained at the beginning of each experiment. NA was added cumulatively and the absolute values of the maximum responses in the facial and basilar arteries were 21 ± 2.1 mN ($n=10$) and 5 ± 1.9 mN ($n=8$), respectively.

During the experiment three subsequent stimulations with 40 mM and 124 mM K⁺ were performed with an interval of 34 min, starting with the first stimulation 64 min after the incubation with ³H-NA, according to the following protocol. Samples were collected 4 times over 1 min periods and the preparation was then immediately stimulated first by a 40 mM and then by a 124 mM K⁺ solution. The exposure time for each solution was 1 min. The vessel was allowed to recover in fresh buffer for 30 min after each stimulation period. When the effect of nifedipine on the ³H-NA release was evaluated, the drug was added to the buffer in all test tubes following the second stimulation by 124 mM potassium.

The efflux of radioactivity from each vessel segment was determined by collecting 2 ml aliquots and mixing it with 10 ml Instagel (Packard). Radioactivity was measured in a liquid scintillation counter (LKB Wallac model 1215) and corrections for quenching were made by means of an automatic external standard according to conventional principles. The counting efficiency was about 45% and the samples were counted for periods of 10 min. The overflow of ³H-NA was expressed as a fraction of the total radioactivity remaining in the tissue before the period of collection, divided by the time (in minutes) during which the transmitter was collected. The total amount

of tritium in the tissue at the onset of each period was determined by adding the radioactivity remaining in the tissue at the end of the experiment to the radioactivity collected. The mean value of the fractional effluxes obtained in 3 consecutive samples immediately before each stimulation period with potassium, was taken as an estimate of the basal fractional outflow. The K⁺-induced release was calculated by subtracting this from the total fractional efflux obtained during K⁺ stimulation. The relative, rather than the absolute values for the fractional outflow of tritium evoked by K⁺, was used for quantification of the observed pharmacological effects. Normalization of the K⁺-induced outflows of tritium was performed by taking the first fractional release induced by 124 mM K⁺ in each experiment as 100%. At the end of the experiments the vascular tissues were dried, weighed and then left to dissolve in 1 ml protosol (New England Nuclear) for 1 h at 50°C, before addition of 10 ml Instagel and subsequent measurement of the tritium content.

Statistical analysis

Statistical analysis of the results was performed by using Student's *t*-test for paired and unpaired data. The 0.05 level of probability was accepted as significant. The results shown in tables, figures and the text are expressed as mean values $\pm$ standard errors of the means.

Solutions and substances

The following buffers were used:

1. Na-Krebs solution composed of (in mM): NaCl 119, KCl 4.6, CaCl₂ 1.5, MgCl₂ 1.2, NaHCO₃ 20, NaH₂PO₄ 1.2, glucose 11.
2. A 124 mM potassium solution containing the same constituents as the Na-Krebs solution except for an equimolar substitution of NaCl for KCl.
3. A 40 mM potassium solution prepared by directly adding KCl to a Na-Krebs solution without correcting for osmotic changes.

The solutions were prepared from analytical grade chemicals. Ascorbic acid (0.5 mM) was included in all buffers used for the release studies in order to reduce oxidation of tritiated noradrenaline (Hughes & Smith 1978).

The tested drugs were *d*, *l*-noradrenaline hydrochloride (Sigma), reserpine (Serpasil®, Ciba-Geigy), phentolamine methanesulfonate (Ciba-Geigy), prazosin (Pfizer) and nifedipine (Adalat®, Bayer).

Prazosin was dissolved in lactic acid (10%) giving a final concentration of 100 μ M. Nifedipine was provided in ampoules (0.1 mg/ml), containing the drug dissolved in ethanol (15%) and polyethylene glycol (15%), and kept dark until used, to avoid light-induced degradation. *d*, *l*-Noradrenaline and phentolamine were dissolved, and the other drugs diluted by physiological saline (0.9%), containing 1 mM ascorbic acid as a protection against oxidation of unstable substances. The drugs were added directly to the organ bath in volumes of 25 μ l and the concentrations given are the final concentrations in the organ bath in moles/liter (M). None of the solvents used did significantly change resting arterial tension or the mechanical response to potassium and noradrenaline *per se*. Calcula-

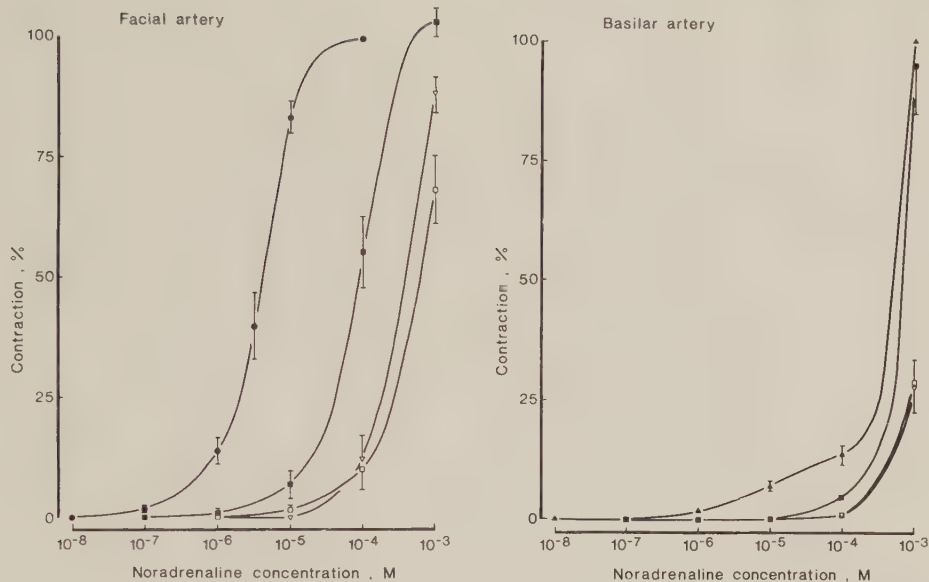


Fig. 4. The effect of phentolamine (■ 1 μ M, □ 10 μ M) and prazosin (▽ 1 μ M) on the log concentration-response curve for NA in isolated rabbit facial and basilar arteries. Control responses to NA were recorded initially in each experiment and the maximum response was taken as 100% (facial artery, ●; basilar artery, ▲). The alpha-ad-

renoceptor blocking agents were given 15 min prior to subsequent NA exposure. Each concentration of antagonist was tested on 5-6 arterial segments obtained from different animals. Only one drug was examined on each segment.

tions of EC_{50} values, the concentration of the drug eliciting half maximum response, were based on the geometrical means.

RESULTS

Contractile responses

Effects of potassium (K^+). After the basilar and facial arteries were suspended in the organ bath, their immediate response was a relaxation when subjected to passive stretches. They never showed any sign of spontaneous contractile activity. Exposure to a K^+ -rich solution (124 mM) invariably contracted the vessel segments. The response consisted of an initial fast increase in tension followed by a sustained tonic contraction. The contractions induced by 124 mM K^+ were reproducible in both types of vessel and the maximum response decayed by less than 10% when 6 repetitive contractions (with an interval of 20 min) were performed in control experiments.

Effects of noradrenaline (NA). Noradrenaline

produced strong contractions in the facial artery, the maximum contractile response amounted to $86 \pm 6\%$ ($n=10$) of that evoked by K^+ (124 mM) and the EC_{50} value was 4.5μ M ($\log EC_{50} = -5.4 \pm 0.06$; $n=10$; Fig. 3). The majority of the basilar arteries tested, responded only to the highest concentrations of NA ($>10 \mu$ M) with small contractions and some arteries failed to respond at all. The potency differed markedly between the two types of vessel, the sensitivity to NA of the facial artery being about 100 times greater than that of the basilar artery (Fig. 3). Since no plateau of the concentration-response curve to NA was obtained in the basilar artery, no E_{max} or EC_{50} value could be calculated. A further increase in tension was observed in facial and basilar arteries previously contracted by potassium (124 mM) upon exposure to 10 μ M (facial artery) and 1 mM (basilar artery) NA. The concentration-response curve to NA was displaced to the right by prazosin (1 μ M) and phentolamine (1 and 10 μ M) in both types of artery (Fig. 4). Prazosin appeared to be more potent than phentolamine.

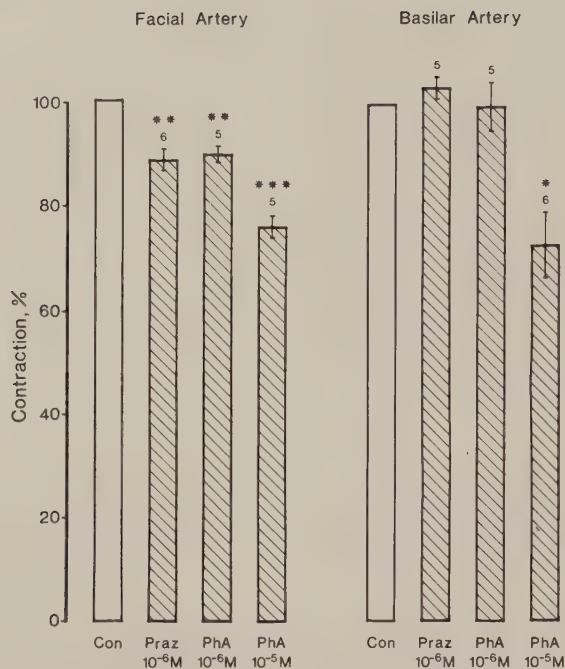


Fig. 5. Depression of K^+ -induced (124 mN) contractions by phentolamine (PhA), 1 and 10 μ M and prazosin (Praz, 1 μ M) in rabbit facial and basilar arteries. The mean of two control contractions (open bars) obtained immediately before application of the alpha-adrenoceptor blockers was taken as 100%, and the absolute values of the maximum responses in facial and basilar arteries were 26 ± 2.4 mN ($n=11$) and 16 ± 1.4 mN ($n=11$), respectively. The antagonists were allowed to equilibrate with the receptor for 15 min before K^+ was added. Figures above the bars indicate the number of arteries (from different animals) examined. * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$, when compared with the controls.

In order to evaluate whether endogenous noradrenaline, released from adrenergic nerves in the vessel wall, contributes to the contraction induced by depolarization of the vessel segments by K^+ excess, arteries were treated with either phentolamine (1 and 10 μ M) or prazosin (1 μ M) for 15 min before exposure to 124 mM K^+ . Prazosin (1 μ M) significantly reduced the response to K^+ of the facial artery by $12 \pm 2\%$ ($n=6$; $p < 0.01$). The corresponding value for phentolamine (1 μ M) was $11 \pm 1\%$ ($n=5$; $p < 0.01$). The K^+ -evoked responses of the basilar artery were unaffected by these concentrations of alpha-adrenoceptor blockers (Fig. 5). However, the highest concentration of phentolamine (10 μ M) reduced the K^+ -induced contractions of both facial

and basilar arteries by $25 \pm 2\%$ ($n=5$; $p < 0.001$) and $27 \pm 7\%$ ($n=6$; $p < 0.02$), respectively. The effects of the drugs were always more pronounced on the tonic component of the K^+ contraction than on the initial rapid phase.

The dependence of the K^+ contraction on extracellular Ca^{++} was investigated by using the Ca^{++} influx inhibitor nifedipine (Fleckenstein 1977). Nifedipine did not cause any change in tension of resting arteries. On the other hand, nifedipine effectively depressed K^+ (124 mM)-induced contractions of both facial and basilar arteries, but never abolished them (Fig. 6). The tonic component of the potassium contraction was reduced at lower concentrations of nifedipine than the rapid component

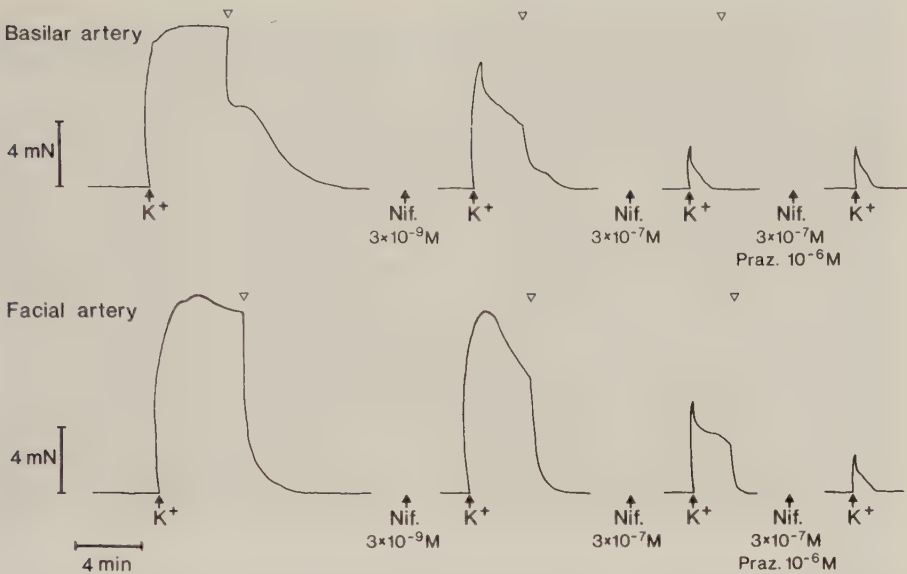
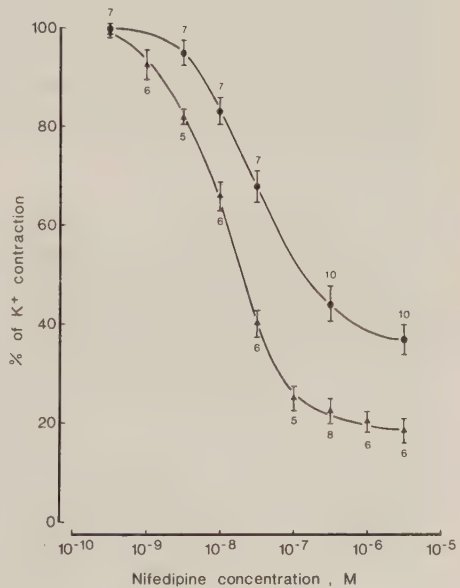


Fig. 6. Typical responses of rabbit basilar (upper) and facial (lower) arteries to K⁺ (124 mM) before and after application of nifedipine (Nif, 3 nM and 0.3 μM) alone, and nifedipine (0.3 μM) in combination with prazosin (Praz, 1 μM). The drugs were added 15 min prior to activation by K⁺. Open triangles indicate when the preparations were rinsed with drug-free Na-Krebs solution.

and was often abolished at the higher concentrations of nifedipine used, leaving a phasic contraction (Fig. 6). The log concentration–response curves for nifedipine in the two arteries are illustrated in Fig. 7. The effect of nifedipine on the K⁺ response was more pronounced in the basilar than in the facial artery as demonstrated by the lower IC₅₀ value (~10 versus ~30 nM, respectively) and greater maximal inhibition, 81 ± 2% (n=6) and 63 ± 3% (n=10), respectively (*p* < 0.001). After the K⁺-induced contractions had been inhibited by nifedipine (0.3 or 3.0 μM), a subsequent contraction in the presence of either prazosin (1 μM) or phenolamine (1 μM) further depressed the K⁺ response

Fig. 7. Concentration–response curves for nifedipine in isolated rabbit basilar (▲) and facial (●) arteries. The mean of two control contractions induced by K⁺ (124 mM) was calculated in the beginning of each experiment and was taken as 100%. The absolute values of these contractions in basilar and facial arteries were 13 ± 1.1 mN (n=22) and 16 ± 1.6 mN (n=25), respectively. Nifedipine was given 15 min before K⁺ was added and the vessels were allowed to relax to the baseline after each K⁺ exposure.



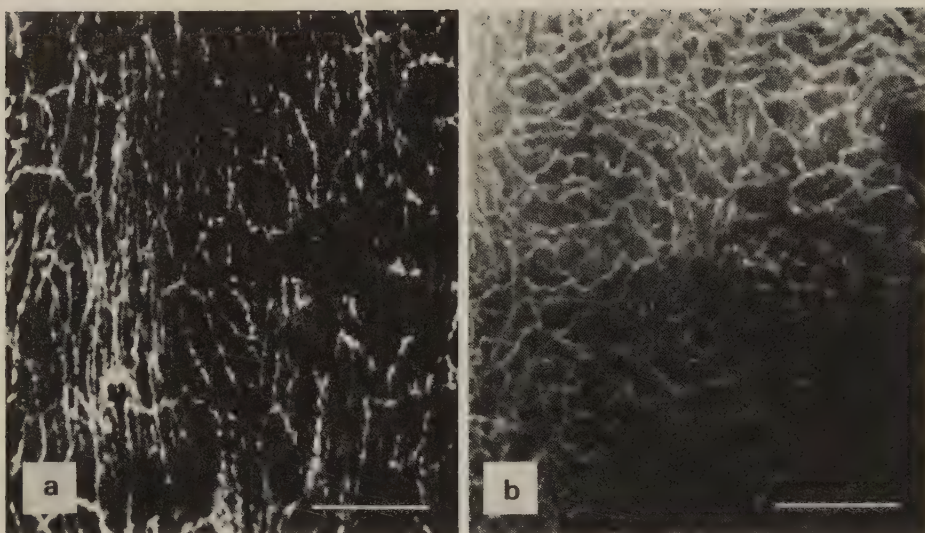


Fig. 8. Fluorescence photomicrographs of the network of adrenergic nerve fibres in the basilar (a) and facial (b) artery from rabbit. The vessels were cut open longitudinally, mounted flat and treated with formaldehyde gas under dry conditions for visualization of the green-fluorescent noradrenaline transmitter. Length of bars = 50 μ m.

of the facial artery (Fig. 6). This effect was not found in the basilar artery. In the presence of the alpha-adrenoceptor blockers, the difference in inhibition observed between the two types of artery by nifedipine alone was abolished. The depressant effect on the facial artery of the alpha-adrenoceptor blocking agents was still obtainable after treatment of this vessel for 12 h in a solution containing 10 μ M reserpine ($n=3$).

Fluorescence histochemistry of adrenergic nerves

A well developed network of green-fluorescent nerve fibres was demonstrated in the walls of the basilar and facial arteries (Fig. 8). The distribution of adrenergic nerves in the vessel wall and the intensity of fluorescence within individual nerve fibres appeared to be similar in the two types of artery examined.

Uptake and release of ^3H -noradrenaline

Further support for the existence of biologically active sympathetic nerves was gained from uptake and release studies. Accumulation of radioactivity was demonstrated in arteries incubated with ^3H -

NA. Subsequent exposure to K^+ -rich media (40 and 124 mM K^+) caused release of the labelled transmitter from the vessel segments. The spontaneous efflux of tritium decayed exponentially during the first half hour after the incubation period and then reached a steady level of fractional release (Fig. 2). Three consecutive stimulations with K^+ were carried out during the latter period with an interval of 34 min. While the absolute values of tritium released by K^+ progressively decayed over the 3 stimulation periods, reflecting a continuous reduction of labelled NA stores, the fractional release remained virtually constant, and did not significantly change from the first to the third stimulation period (Fig. 9). The reproducibility of the fractional effluxes offered the possibility to use the initial two stimulation periods as controls for the third one, which could be subjected to pharmacological manipulations.

Data obtained in control experiments from basilar and facial arteries are given in Table 1. As can be seen, the basal fractional effluxes of tritium differed significantly between the basilar and facial arteries ($p<0.001$). A small but significant release of labelled noradrenaline was produced by 40 mM K^+ in both

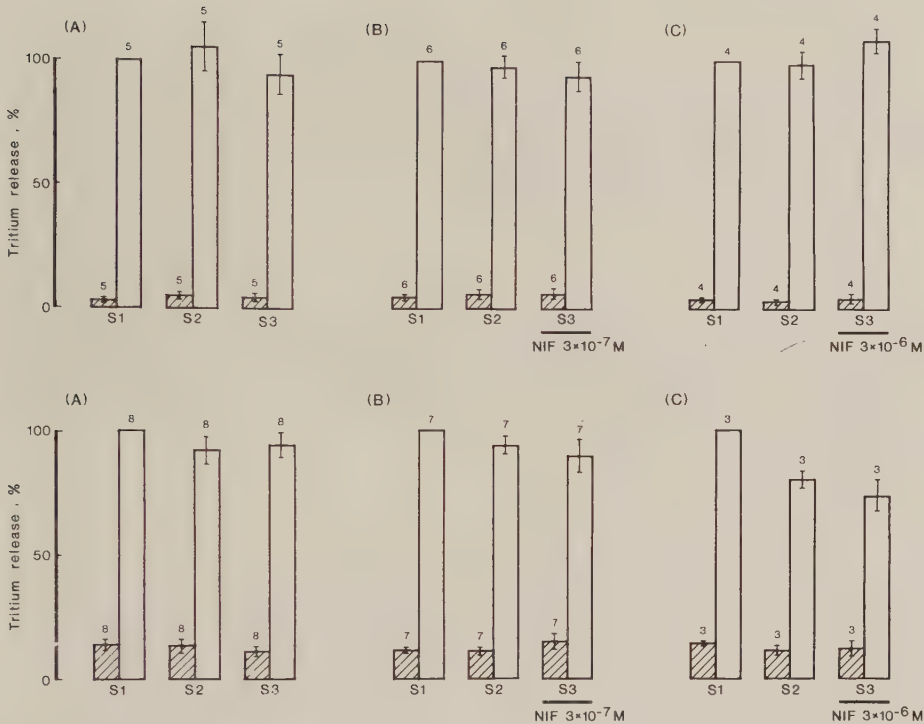


Fig. 9. The effect of nifedipine (NIF) on K^+ -evoked release of 3H -NA from isolated rabbit facial (upper) and basilar (lower) arteries. The preparations were consecutively exposed to solutions containing 40 mM (hatched bars) and 124 mM (open bars) K^+ , and 3 subsequent stimulations (S1–S3) were performed with an interval of 34 min. The exposure time for each solution was 1 min. The increased fractional release evoked by K^+ was calculated by subtracting the spontaneous from the total fractional overflow of tritium collected in each sample. The fractional 3H -efflux obtained during the first exposure to 124 mM K^+ (S1) was taken as 100%. Control experiment (A); effect of 0.3 μM nifedipine on S3 (B); effect of 3.0 μM nifedipine on S3 (C). Nifedipine (0.3 and 3.0 μM) was supplied to the buffer 34 min before S3. Figures above the bars indicate the number of experiments performed.

types of artery ($p < 0.001$). The largest release was obtained after exposing the arteries to 124 mM K^+ . Under these conditions the fractional effluxes from the basilar and facial arteries were estimated to $6.6 \pm 0.6\%$ ($n = 18$; $p < 0.001$) and $4.0 \pm 0.3\%$ ($n = 15$; $p < 0.001$), respectively. Thus, a significantly greater fractional release of 3H -NA (about 65%; $p < 0.001$) was observed in the basilar artery than in the facial artery. However, this difference was abolished when the efflux of 3H -NA was calculated as a percentage of the basal fractional efflux or in counts per min (CPM) per mg tissue (Table 1).

In order to distinguish between neuronally and extraneuronally located uptake and subsequent re-

lease of tritiated noradrenaline, 10 μM desipramine was added to the buffer 60 min prior to incubation with labelled NA and kept present throughout the experiment. This treatment attenuated the 3H -NA uptake by 85% (basilar artery; $p < 0.02$) and 83% (facial artery; $p < 0.001$), and reduced the transmitter release induced by 124 mM K^+ (in all three consecutive stimulations) to less than 5% ($p < 0.001$) of that observed in untreated vessels (Table 2). The desipramine-sensitive noradrenaline uptake, calculated by subtracting the uptake observed in desipramine-treated vessels from that seen in the absence of the drug, was used as an estimate of the nerve-mediated transmitter accumu-

Table 1. The effect of K^+ on the 3H -NA overflow in rabbit facial and basilar arteries

Releasing agent	I Fractional release, %		I Fractional release, % Desipramine treated (10 μ M)		II Release, $\% \times 10^{-2}$	
	Facial artery	Basilar artery	Facial artery	Basilar artery	Facial artery	Basilar artery
Basal efflux	0.43 ± 0.03^b n=15	0.80 ± 0.03^a n=18	0.77 ± 0.06^c n=4	0.9 ± 0.19 n=4		
40 mM K^+	$0.13 \pm 0.03^{b,d}$ n=15	$0.7 \pm 0.11^{a,d}$ n=18	0.04 ± 0.02^c n=4	0.00 ± 0.03^c n=4	$0.34 \pm 0.07^{b,d}$ n=15	$0.9 \pm 0.14^{a,d}$ n=18
124 mM K^+	$4.0 \pm 0.32^{b,d}$ n=15	$6.6 \pm 0.56^{a,d}$ n=18	0.16 ± 0.06^c n=4	0.07 ± 0.04^c n=4	10.0 ± 0.95^d n=15	8.6 ± 0.91^d n=18

The efflux of 3H -NA is expressed: (I) As a fraction (in %) of the total tissue radioactivity released per min; (II) as a percentage of the basal fractional release; (III) in CPM per mg tissue (dry weight). All the data have been taken from the first stimulation period (S 1). The exposure time for each K^+ solution was 1 min. The K^+ -evoked outflows were calculated by subtracting the spontaneous release from the total outflow of tritium collected in each sample. Desipramine was added 60 min before the start of the incubation period with 3H -NA and kept present throughout the expt. n = number of expts.

^a Significantly different from the facial artery.

^b Significantly different from the basilar artery.

^c Significantly different from the corresponding untreated vessels.

^d Significantly increased above the basal efflux.

lation. As can be seen in Table 2, both types of vessel were equally efficient in accumulating 3H -NA.

Nifedipine in concentrations which produced a maximum inhibition of K^+ -induced contractions (0.3 μ M and 3 μ M) failed to reduce the spontaneous or K^+ -evoked release of 3H -NA from basilar and facial arteries (Fig. 9). The data from these experiments are summarized in Table 3.

DISCUSSION

The present study clearly shows that nifedipine effectively inhibits potassium (K^+)-evoked contractions in isolated basilar and facial arteries from rabbit. This effect is probably not mediated through a reduction of K^+ -induced efflux of endogenous nor-adrenaline (NA) from adrenergic nerves in the vessel walls and subsequent, cessation of postsynaptic alpha-adrenoceptor stimulation for two main reasons:

1. Nifedipine in concentrations which effectively inhibited contractions elicited by K^+ , failed to significantly affect K^+ -evoked release of 3H -NA (Fig. 9).

2. The alpha-adrenoceptor blocking agents phenolamine and prazosin in concentrations that markedly suppressed the response to exogenous NA had only minimal effects on K^+ -evoked contrac-

tions (Fig. 5), indicating that the participation of NA in maintaining the contractile response to K^+ is either small (facial artery) or negligible (basilar artery). The extremely high concentrations of NA necessary to produce even the smallest contraction in the basilar artery also support this interpretation.

The low sensitivity to NA and the atypical shape of the log concentration-response curve for NA in the basilar artery compared with the facial artery (Fig. 3), underline the difference between cerebral and peripheral vessels in response to catecholamines and confirm the unusual properties of the alpha-adrenoceptor in the rabbit basilar artery reported by others (Bohr et al. 1961, Toda & Fujita 1973, Duckles & Bevan 1976).

A well developed network of adrenergic nerves has previously been demonstrated in the vessel walls of basilar arteries from rabbit (Peerless & Yasargil 1971, Edvinsson & Owman 1977). The present study is in agreement with these results and also shows that a similar arrangement of sympathetic nerves exists in the facial artery (Fig. 8). Incubation of the basilar and facial artery in 3H -NA showed that these vessels were able to accumulate the transmitter (Edvinsson & Owman 1977, Duckles 1980) and to release it upon stimulation with high potassium solutions. The uptake and subsequent release of 3H -NA were significantly reduced by desipramine (10 μ M), an inhibitor of the NA uptake into adrenergic nerves (Leitz & Stefano 1970),

III

Release, CPM $\times 10^{-2}$ /mg

Facial artery	Basilar artery
5.5 $\pm$ 0.46 ^b n=15	11 $\pm$ 1.4 ^a n=24
2.2 $\pm$ 0.50 ^{b, d} n=14	10 $\pm$ 1.6 ^{a, d} n=23
59 $\pm$ 8.1 ^d n=15	73 $\pm$ 10 ^d n=20

Table 2. Reduction of ³H-NA uptake by desipramine (10 μ M) in rabbit facial and basilar arteries

Desipramine was added 60 min before the vessels were incubated with ³H-NA and kept present throughout the experiment. The preparations were incubated with ³H-NA for 60 min and then washed in 3 ml Krebs buffer for 10 min (3 times), before the tritium content of the tissues was assayed. The uptake of ³H-NA is expressed in counts per min (CPM) per mg tissue (dry weight)

	Uptake, CPM $\times 10^{-2}$ /mg	
	Facial artery	Basilar artery
Controls	1 728 $\pm$ 218 n=12	1 623 $\pm$ 301 n=7
Desipramine treated (10 μ M)	294 $\pm$ 70 ^a n=5	248 $\pm$ 67 ^a n=4

^a Significantly different from the corresponding controls.

which strongly suggests that these functions were mediated through sympathetic nerves. In spite of an extensive sympathetic innervation, electrical field stimulation of the rabbit basilar artery has been shown to produce only small responses (Lee et al. 1976) as compared with peripheral vessels (Toda & Fujita 1973). This observation may be partly explained by the fairly insensitive postsynaptic alpha-adrenoceptor present in the basilar artery (Duckles & Bevan 1976). At the moment, no definite answer is available to the question of the func-

tional significance of the sympathetic nerves located in the rabbit basilar artery.

A greater susceptibility of rabbit and canine cerebral than of peripheral arteries to the Ca⁺⁺ influx inhibitors verapamil, diltiazem, nifedipine and nimodipine has previously been suggested (Hayashi & Toda 1977, Allen & Banghart 1979, Shimizu et al. 1980, Towert & Kazda 1980). The present study indicates that nifedipine displays a similar selectivity for cerebral arteries in rabbits (Fig. 7). This difference may reflect a greater utilization of extracellular Ca⁺⁺ for contraction in cerebral than in peripheral vessels. However, it may also be attributed

Table 3. Effect of nifedipine on basal and K⁺-evoked release of ³H-NA from rabbit facial and basilar arteries

The effluxes of ³H-NA are expressed as fractions (in %) of the total tissue radioactivity released per min. The increased release induced by K⁺ was calculated by subtracting the spontaneous from the total fractional outflow collected in each sample. The control values refer to the fractional release obtained during the second stimulation period (S2). Nifedipine (NIF) was added 34 min before the last stimulation period (S3)

Releasing agent	Fractional release, %							
	Facial artery				Basilar artery			
	Control	NIF 0.3 μ M	Control	NIF 3.0 μ M	Control	NIF 0.3 μ M	Control	NIF 3.0 μ M
Basal efflux	0.33 $\pm$ 0.04 n=6	0.28 $\pm$ 0.04 n=6	0.33 $\pm$ 0.04 n=4	0.28 $\pm$ 0.03 n=4	0.80 $\pm$ 0.07 n=8	0.9 $\pm$ 0.10 n=8	0.8 $\pm$ 0.14 n=3	0.95 $\pm$ 0.02 n=3
40 mM K ⁺	0.22 $\pm$ 0.08 n=6	0.20 $\pm$ 0.08 n=6	0.12 $\pm$ 0.05 n=4	0.2 $\pm$ 0.11 n=4	0.6 $\pm$ 0.10 n=8	0.70 $\pm$ 0.09 n=8	0.9 $\pm$ 0.17 n=3	1.0 $\pm$ 0.30 n=3
124 mM K ⁺	3.8 $\pm$ 0.62 n=6	3.6 $\pm$ 0.60 n=6	3.6 $\pm$ 0.22 n=4	4.0 $\pm$ 0.38 n=4	5.9 $\pm$ 0.93 n=8	6 $\pm$ 1.1 n=8	6.5 $\pm$ 0.66 n=3	6.1 $\pm$ 0.89 n=3

to dissimilarities in the contractile effect elicited by NA released from perivascular nerves. Several investigations have shown that NA-elicited contractions in peripheral arteries are less sensitive to Ca^{++} antagonists and to deprivation of Ca^{++} from the extracellular medium than K^{+} -evoked contractions (Hiraoka *et al.* 1968, Hudgins & Weiss 1968, Peiper *et al.* 1971, Lederballe Pedersen *et al.* 1978, Mikkelsen *et al.* 1979, van Breemen & Siegel 1980), a difference explainable by a greater ability of NA to mobilize intracellular Ca^{++} for contraction than K^{+} . In the present study phentolamine and prazosin further enhanced the nifedipine-produced inhibition of K^{+} -induced contraction in facial but not in basilar arteries (Fig. 6). On the basis of these findings the possibility was raised that endogenous NA activated postsynaptic α -adrenoceptors in the facial artery resulting in a contraction resistant to inhibition by nifedipine. However, this was probably not the case since the effect of the α -adrenoceptor blocking agents was still observed after depletion of NA stores with reserpine.

Invariably, the sustained tonic component of the K^{+} contraction was more sensitive and depressed by lower concentrations of nifedipine than the initial rapid component (Fig. 6). It may be argued whether the source of Ca^{++} in the initial fast phase is mainly extracellular. Intracellular storage sites may provide the activator Ca^{++} necessary for the initial fast contraction, as has been suggested for the NA-induced contraction of the rabbit ear artery and aorta (Steinsland *et al.* 1973, Deth & van Breemen 1974). Influx of Ca^{++} from the extracellular medium or from an extracellular surface membrane associated Ca^{++} pool through ROCs (Bolton 1979), which are not inhibited by nifedipine, may also explain the persisting phasic contraction observed after nifedipine treatment of basilar and facial arteries.

Parallels have been drawn between the mobilization and movements of Ca^{++} during contraction of vascular smooth muscle and transmitter efflux from nerve endings. Both processes have been shown to depend on the access to extracellular Ca^{++} (Boullin 1967, Hudgins & Weiss 1968, Kirpekar & Wakade 1968, van Breemen & Siegel 1980). Perfusion of the cat spleen with Ca^{++} -deficient solutions attenuated adrenergic transmitter release induced by K^{+} or electrical stimulations of the splanchnic nerve (Garcia *et al.* 1976), and most vascular smooth muscle contractions are suppressed in Ca^{++} -deprived

media (Hudgins & Weiss 1968, van Breemen & Siegel 1980).

Several multivalent cations documented to interfere with smooth muscle contraction, appear also to have effects on transmitter release. Thus, externally applied strontium and barium can replace Ca^{++} in contraction of smooth muscle (Daniel 1963, Toda 1973) and in transmitter efflux induced by depolarization (Boullin 1967, Kirpekar & Misu 1967). On the other hand, lanthanum, magnesium, cadmium, manganese, cobalt, and nickel are unable to replace calcium, but rather they antagonize the effects of calcium on transmitter release and contraction (Boullin 1967, Sullivan & Briggs 1968, van Breemen *et al.* 1972, Kirpekar *et al.* 1970, 1972, Toda 1973, Altura & Altura 1978). Ca^{++} -antagonistic drugs, which are believed to interfere with potential sensitive slow Ca^{++} channels (Fleckenstein 1977), have recently been used to elucidate and characterize Ca^{++} -requiring processes. The present study demonstrates that nifedipine, which belongs to this group of drugs, in concentrations that produce a maximum inhibition of K^{+} induced contractions in rabbit cerebral and extracranial arteries, failed to alter transmitter release from perivascular sympathetic nerves in these vessels (Fig. 9). A greater susceptibility to nifedipine of the smooth muscle cell than of the adrenergic nerve endings might reflect (assuming a constant Ca^{++} -channel density) a greater surface to volume ratio in the nerves, this making the nerve endings less affected by drugs interfering with the transport of Ca^{++} across the synaptic membrane. However, potential sensitive Ca^{++} channels in the nerve endings with properties different from those operating in smooth muscle, could also explain the experimental findings.

Recently, Ichida *et al.* (1980) demonstrated a reduced K^{+} -stimulated $^{45}\text{Ca}^{++}$ uptake by synaptosomes from rat brain in the presence of verapamil. However, the concentrations of Ca^{++} -antagonist employed in this study ($\geq 1 \mu\text{M}$) and in a number of others (Starke & Schumann 1973, Vargas *et al.* 1977, Göthert *et al.* 1979) by far exceed those having relaxant effect on vascular smooth muscle (Schumann *et al.* 1975, Fleckenstein 1977, Mikkelsen *et al.* 1979, Shimizu *et al.* 1980). Our results are in keeping with the findings of Haeusler (1972) who concluded that the action of verapamil did not seem to involve adrenergic nerve terminals, as the drug did not influence the Ca^{++} -dependent release of noradrenaline from the isolated heart produced

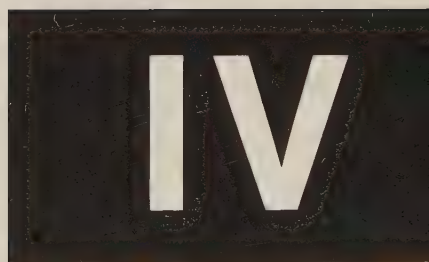
either by sympathetic nerve stimulation, acetylcholine or K^+ . It was also shown that high concentrations of verapamil reduced both acetylcholine- and K^+ -evoked antidromic discharges of the inferior cardiac nerve, indicating a local anaesthetic effect which could account for the suppressed release of 3H -NA observed by some investigators. We conclude that nifedipine, in concentrations which effectively inhibit K^+ -evoked contractions in isolated rabbit cerebral and extracranial arteries, does not influence K^+ -induced release of 3H -NA from intramural postganglionic sympathetic nerves, but rather act directly on the vascular smooth muscle cells.

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**CHARACTERIZATION OF TWO DIFFERENT CALCIUM ENTRY PATHWAYS
IN SMALL MESENTERIC ARTERIES FROM RAT**

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Running title: Ca^{2+} entry pathways in rat mesenteric arteries

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ABSTRACT

Högestätt, E.D.: Characterization of two different calcium entry pathways in small mesenteric arteries from rat.

The effects of Ca^{2+} removal, nifedipine, and La^{3+} on contractions induced by 124 mM K^{+} and 10 μM noradrenaline (NA) were investigated in small mesenteric arteries from rat. Ring segments of the arteries were suspended between two steel wires in a 2.5 ml muscle bath, and the mechanical activity recorded "isometrically". The tonic components of the contractile responses to both K^{+} and NA were critically dependent on the presence of Ca^{2+} in the bath solution. Nifedipine effectively relaxed K^{+} -contracted arteries, whereas those activated by NA were considerably less affected by the drug. Application of NA to arteries depolarized by K^{+} in the presence of nifedipine induced sustained tonic contractions, which were only approximately 20% smaller than those elicited by NA in "standard" Krebs solution, implicating pharmacomechanical coupling. Unlike nifedipine, La^{3+} inhibited K^{+} - and NA-induced contractions to approximately the same extent. Re-application of Ca^{2+} to " Ca^{2+} -depleted" preparations exposed to K^{+} and/or NA induced concentration-dependent contractions. The experimental results suggested that the effects of K^{+} and NA on the membrane permeability to Ca^{2+} were additive. The Ca^{2+} -induced contractions were more inhibited by nifedipine in K^{+} -depolarized than in NA-exposed arteries. It is concluded that K^{+} and NA utilize partly different Ca^{2+} entry pathways to increase the myoplasmic Ca^{2+} concentration in rat mesenteric arteries. Whereas K^{+} seems to promote the influx of Ca^{2+} by activation of Ca^{2+} channels sensitive to the membrane potential, the nature of the receptor-operated Ca^{2+} entry pathway remains to be established.

Key words: rat mesenteric arteries - excitation-contraction coupling - potassium contraction - noradrenaline contraction - extracellular calcium - nifedipine - lanthanum

INTRODUCTION

Influx of extracellular Ca^{2+} is well known to play an essential role in the activation of vascular smooth muscle (VSM). Ca^{2+} has been suggested to enter the VSM cells in several ways: (1) via specific channels activated by stimulants, (2) in exchange for Na^+ by a hypothetical $\text{Na}^+-\text{Ca}^{2+}$ exchange carrier and (3) by a less well defined "passive leak" of the ion across the cell membrane, accounting for the Ca^{2+} uptake in the resting muscle (see, e.g., Bolton 1979, van Breemen et al. 1979).

Organic " Ca^{2+} -entry blockers", such as nifedipine, verapamil and diltiazem, are considered to reduce the stimulation-induced influx of Ca^{2+} into "excitable" cells by blocking specific membrane channels for Ca^{2+} (Fleckenstein 1977, Bolton 1979, Nayler and Poole-Wilson 1981, Triggler 1983). Studies of the effects of these agents on different smooth muscle tissues have led several research groups to postulate the existence of different Ca^{2+} entry pathways in the cell membrane. Golenhofen and Hermstein (1975) presented evidence of two different Ca^{2+} activation systems (both dependent on extracellular Ca^{2+}) in the isolated guinea-pig portal vein, one associated with spike activity and inhibited by verapamil and D 600 (P system) and another spike-free mechanism resistant to these drugs (T system) (see also Golenhofen 1981). Based on unidirectional $^{45}\text{Ca}^{2+}$ flux measurements in the rabbit aorta, Meisheri et al. (1981) provided direct evidence for the existence of two separate Ca^{2+} entry pathways in the cell membrane, one activated by K^+ depolarization and sensitive to D 600, the other stimulated by α -adrenoceptor activation and relatively resistant to D 600. However, these studies gave no suggestion as to the nature of the different pathways.

Bolton (1979) offered an explanation for the selective action of Ca^{2+} -entry blockers by postulating that membrane depolarization and receptor occupation by agonists may activate two different types of Ca^{2+} channel, differing in their sensitivity to Ca^{2+} -entry blockers. These channels were termed potential- and receptor-operated channels (POCs, ROCs), respectively. However, the general validity of the assumption that ROCs are less affected by Ca^{2+} -entry blockers than POCs has recently been questioned (see

Cauvin et al. 1983) on the basis of experiments showing that in some VSM preparations contractions induced by NA are more potently inhibited by Ca^{2+} -entry blockers than K^{+} -induced contractions (Bevan 1981, Walus et al. 1981, Cauvin et al. 1982). Some authors have even proposed alternative models for the receptor-operated pathway to account for the resistance of agonist-induced contractions to these agents (Casteels & Droogmans 1981, Cauvin et al. 1983).

In order to obtain more information on the mechanisms involved in depolarization- and agonist-induced contractile responses, the effects of Ca^{2+} removal and nifedipine on K^{+} - and NA-induced contractions were examined in isolated small mesenteric arteries from rat. For sake of comparison, the effects of La^{3+} on the contractile responses to these stimulants were also studied. Evidence will be presented suggesting that K^{+} depolarization and stimulation of postjunctional α -adrenoceptors utilize partly different Ca^{2+} entry pathways to increase the myoplasmic Ca^{2+} concentration.

MATERIALS AND METHODS

Vascular preparation

Second generation of branches originating from the superior mesenteric artery (ca 200 μm in diameter) were removed from adult Sprague-Dawley rats (200 - 300 g) of either sex. The animals were purchased from Alab (Stockholm, Sweden) and killed by decapitation. The excised arteries were placed in cold (8-12 $^{\circ}\text{C}$) "standard" Krebs solution (for composition, see below) and cleaned from surrounding adipose tissue. The vessels were either used immediately for experimentation or stored in a refrigerator (8 - 12 $^{\circ}\text{C}$) for no longer than 24 h.

6-hydroxydopamine treatment

In order to destroy peripheral adrenergic nerv endings, 100 mg/kg of 6-hydroxydopamine HBr (6-OHDA, Sigma) was injected into a tail vein 24 hours before sacrifice. 6-OHDA was dissolved in ice cold 1 % ascorbic acid, giving a final concentration of 100 mg/ml. The solution with 6-OHDA was kept on ice and protected from light until injected.

Recording of mechanical responses

Ring segments of the arteries, approximately 1 to 2 mm long, were suspended between two stainless steel wires (50 - 100 μm diameter) in a temperature controlled (37 $^{\circ}\text{C}$) small volume muscle bath (2.5 ml), as previously described (Högestätt et al. 1983). Mechanical activity was recorded "isometrically" by means of a Grass Instruments FT03C force-displacement transducer, connected to one of the two steel wires. The signal from the transducer was displayed on a Grass Instruments model 7D polygraph. The muscle bath contained standard Krebs solution, which was continuously bubbled with 95 % O_2 and 5 % CO_2 , resulting in a pH of approximately 7.4. Tris-buffered solutions (for composition, see below) were bubbled with 100 % O_2 . During an equilibration period of 60 min, the resting tension of the vascular preparation was adjusted to approximately 2 mN.

Experimental procedures

After the accommodation period, the contractile capacity was assayed by repeatedly exposing the arterial segment either to 10

μM NA or to an isotonic 124 mM K^+ solution (for composition, see below). These concentrations of K^+ and NA have previously been shown to elicit almost maximum contractions in rat mesenteric arteries, as determined from concentration-response curves for the stimulants (Mulvany et al. 1982). Preparations which failed to produce reproducible contractions were discarded. Contractions were considered reproducible when the maximum tension of two consecutive contractions differed by less than 10 %. The mean amplitude of these responses then served as an internal reference.

Contractile responses to Ca^{2+} in K^+ -depolarized and/or NA-exposed arteries were obtained according to the following protocol. After approximately 20 min in " Ca^{2+} -free" standard Krebs solution (containing 10 μM EGTA), the preparations were repeatedly exposed to a Ca^{2+} -free, 124 mM K^+ solution (containing 10 μM EGTA) and/or NA (10 μM) until the contractile response was reduced to a minimum. The vascular segments were then left in contact with the stimulant(s) for approximately 10 min before Ca^{2+} was re-introduced. Concentration-response relationships for Ca^{2+} were obtained by cumulatively adding Ca^{2+} in the following concentrations (in mM): 0.01, 0.03, 0.1, 1.3, 2.3, 4.3. Consecutive, cumulative applications of Ca^{2+} were separated by a 20-min recovery period in standard Krebs solution. All experiments with LaCl_3 were performed in Tris-buffered solutions. LaCl_3 was added to the preparations 15 to 20 min before K^+ and NA, and the bath solution replaced once during this time (after approximately 10 min).

When indicated, prazosin (0.1 μM) was included in the bath solution in order to remove the influence of neuronally released NA on the VSM cells. As shown previously (Högestätt & Andersson 1984b), this concentration of prazosin abolished the contractile response to exogenously applied NA (0.1-10 μM).

Buffer solutions

1. The standard Krebs solution was composed of (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, (+)-glucose 6.0.

2. The isotonic 124 mM K^+ solution was prepared by substituting all NaCl in the standard Krebs solution with equimolar amounts of KCl.

3. Tris-buffered solutions were prepared by replacing the $NaHCO_3$ and NaH_2PO_4 in the buffers described above with 15 mM NaCl and 5 mM Tris (Trishydroxymethyl-aminomethane) (Sigma). The pH was adjusted to approximately 7.4 at 37°C by adding small volumes of HCl.

4. Ca^{2+} -free solutions were obtained by omitting $CaCl_2$ from the buffers described above and replacing it with either 10 μ M or 1 mM EGTA (ethyleneglycol bis (beta-aminoethyl ether)-N,N'-tetraacetic acid) (Sigma).

Analytical grade chemicals and redistilled water were used for preparing all solutions.

Substances

($\pm$)-Noradrenaline HCl (Sigma) was dissolved and diluted in 0.9 % NaCl (containing 1 mM ascorbic acid). Prazosin (Pfizer) was dissolved in 1 mM HCl, giving a final concentration of 0.1 mM, and then further diluted in water. Nifedipine (Bayer) was provided in ampoules (0.1 mg/ml), containing the drug dissolved in a mixture of ethanol (150 mg/ml), polyethylene glycol (150 mg/ml), and water. Further dilutions were made in 0.9 % NaCl. Nifedipine was kept dark until used to minimize light-induced degradation. $LaCl_3$ (Sigma) was dissolved and diluted in water. Stock solutions of the different substances were stored at -70 °C until used. Fresh dilutions were made daily. All substances, including $CaCl_2$, were added directly to the muscle bath in volumes of 5 to 25 μ l. The concentrations reported are the calculated final bath concentrations, assuming a complete dissociation of the different salts.

Statistics

Statistical analysis of the results were performed by using Student's t-test (two-tailed) for unpaired or paired data when applicable. A probability value of 0.05 was accepted as significant for differences between groups of data. The results shown in the table, figures, and the text are expressed as mean $\pm$ SEM;

n denotes the number of arterial segments examined (all from different animals). The individual concentration-response curves were drawn by hand, and the EC₅₀ or IC₅₀ values were determined graphically by linear interpolation for each curve.

RESULTS

Effects of Ca^{2+} removal and nifedipine on K^{+} - and NA-induced contractions

K^{+} (124 mM), either in the absence or presence of 0.1 μM prazosin, and NA (10 μM) induced stable and reproducible contractions in the isolated rat mesenteric artery (RMA). The contractions could usually be divided into an early fast component and an ensuing slow (tonic) component. Removal of Ca^{2+} from the bath solution during the course of an established K^{+} - or NA-induced contraction caused an immediate relaxation of the preparation (Fig 1). After 5 min in Ca^{2+} -free medium (containing 1 mM EGTA), the active tension was reduced by 97 % irrespective of the stimulant used to elicit contraction. However, nifedipine (30 nM) produced a larger relaxation of K^{+} - than of NA-activated arteries (Fig 2). Notably, the nifedipine-induced relaxation of K^{+} -contracted vessels was considerably faster in preparations previously exposed to prazosin (0.1 μM) than in those which were not. The results from these experiments are summarized in Fig 3. It is evident that although the tonic component of the contractile response to NA was almost completely abolished in Ca^{2+} -free medium, it was relatively resistant to nifedipine.

Response to NA in K^{+} -depolarized arteries

Addition of 10 μM NA to arteries previously contracted by 124 mM K^{+} further increased the active tension by 17 ± 3 % (n=5). However, in arteries removed from 6-OHDA treated rats, NA increased the K^{+} -induced contraction by 69 ± 11 % (n=6). Arteries incubated with 0.3 μM nifedipine responded to K^{+} with a transient contraction, which stabilized after approximately 10 min at a level slightly above the baseline tension (Fig 4). Application of NA under these conditions induced a sustained tonic contraction, which was only 22 ± 3 % (n=8) smaller than that elicited by NA in drug-free standard Krebs solution (Fig 4). The contractions thus obtained were rapidly reversed by removing Ca^{2+} from the bath solution (Fig 4).

Concentration-response relationship for Ca^{2+} in K^{+} -depolarized and/or NA-exposed arteries

Ca^{2+} (0.01 - 4.3 mM) induced concentration-dependent contractions in arteries presoaked in Ca^{2+} -free medium and exposed to either 124 mM K^{+} (in the absence or presence of 0.1 μM prazosin), 10 μM NA, or to both 124 mM K^{+} and 10 μM NA (Fig 5, Table 1). In vessels exposed to both K^{+} and NA and in those incubated with NA alone, application of Ca^{2+} in concentrations higher than 1 mM usually reduced the active tension (Fig 5). Notably, exposure to 4.3 mM Ca^{2+} caused irreversible damage to arteries bathing in a medium containing both K^{+} and NA. Prazosin (0.1 μM) significantly reduced the maximum contractile response to Ca^{2+} (4.3 mM) in K^{+} -depolarized arteries by approximately 36 % (Table 1), but only tended to shift the concentration-response curve for Ca^{2+} to the right (Fig 5); the EC_{50} values for Ca^{2+} did not differ significantly between the two groups of artery. However, the Ca^{2+} concentration eliciting half maximum contraction was approximately three times lower in NA-exposed than in K^{+} -depolarized arteries. The graded responses to Ca^{2+} were not significantly enhanced by increasing the NA concentration in the bath solution from 10 μM to 0.1 mM ($n=4$). When added simultaneously, K^{+} and NA further increased the Ca^{2+} sensitivity of the RMA, as compared with separate applications of the stimulants (Fig 5, Table 1). Moreover, the maximum contractile response to Ca^{2+} was 37 % smaller in arteries depolarized by K^{+} (in the presence of 0.1 μM prazosin) than in those exposed to both K^{+} and NA (Table 1).

Effects of nifedipine on Ca^{2+} -induced contractions in K^{+} -depolarized or NA-exposed arteries

The effect of nifedipine (30 nM and 0.3 μM) on the contractile response evoked by re-application of 1.5 mM Ca^{2+} to " Ca^{2+} -depleted" arteries exposed either to 124 mM K^{+} (in the presence of 0.1 μM prazosin) or 10 μM NA is shown in Fig 6. As can be seen, nifedipine preferentially inhibited the Ca^{2+} -induced contraction in the preparation depolarized by K^{+} . The drug also effectively suppressed graded contractions induced by adding increasing concentrations of Ca^{2+} (0.01 - 4.3 mM) to K^{+} -depolarized vessels (exposed to 0.1 μM prazosin), and in the presence of

0.3 μM nifedipine, the response to Ca^{2+} was fully abolished (Fig 7, upper panel). However, the Ca^{2+} -induced contractions were found to be comparatively less affected by nifedipine in NA-exposed arteries (Fig 7, lower panel). Notably, the inhibition produced by nifedipine was larger the lower the Ca^{2+} concentration used to elicit contraction.

Effects of La^{3+} on K^{+} - and NA-induced contractions

Exposure to Tris buffer did not appreciably alter the contractile response to 124 mM K^{+} (in the presence of 0.1 μM prazosin) and 10 μM NA (n=9). Addition of La^{3+} to this solution effectively reduced K^{+} - and NA-induced contractions. Fig 8 shows tracings of contractile responses elicited by the two stimulants before and after application of 10 μM La^{3+} . In contrast to the response to NA, which decayed after an initial peak, the K^{+} -induced contraction was well maintained and usually slowly increased over a 60 min period. The maximum contractile response obtained during the first 5 min of exposure to K^{+} or NA was used for measuring the inhibition produced by La^{3+} , and the results are shown in Fig 9. As can be seen, La^{3+} was almost equipotent as an inhibitor of K^{+} - and NA-induced contractions; a significant difference was observed only with one concentration of La^{3+} (0.1 mM). The La^{3+} concentration producing half maximum inhibition was approximately 3 μM for contractions induced by both stimulants. The inhibition produced by La^{3+} was only partly reversible.

DISCUSSION

Contribution of endogenous NA to the K^+ -induced contraction

K^+ and NA are widely used stimulants in studies of the excitation-contraction coupling process in VSM. Apart from directly activating the VSM cells, high K^+ solutions may also cause neuronal transmitter release. Indeed, it was found that approximately 30 to 40 % of the tonic component of the K^+ -induced contraction in the RMA was attributed to stimulation of postjunctional α -adrenoceptors by NA released from adrenergic nerve endings (Whall et al. 1983). In the present study, the maximum contractile response induced by re-addition of Ca^{2+} to Ca^{2+} -depleted arteries depolarized by K^+ was 36 % smaller in vessels pretreated by prazosin than in those which were not, suggesting that endogenously released NA plays a significant role also in these contractions.

When the adrenergic component was reduced either by exposing the artery to phentolamine or prazosin, or by using vessels chemically denervated by 6-OHDA, K^+ induced a biphasic contraction, composed of an initial fast component and an ensuing slow (tonic) component, which were separated by a small transient relaxation (Mulvany et al. 1982, Whall et al. 1983). A similar biphasic course of the response to K^+ depolarization has previously been observed in the isolated rat basilar artery and interpreted as reflecting influx of Ca^{2+} via two different types of POC, differing in their kinetic properties (Högestätt & Andersson 1984a). This explanation may apply also to the biphasic contraction elicited by K^+ in the RMA.

Does NA induce contraction by membrane depolarization?

It is well established that NA can stimulate contraction in certain VSM preparations in a manner which is not associated with changes of the membrane potential (Somlyo & Somlyo 1968, Johansson & Somlyo 1980). Evidence for this has been obtained both directly from simultaneous recordings of membrane potential and mechanical activity, and indirectly from studies of the effects of agonists on K^+ -depolarized muscles (see, e.g., Bolton 1979). Exposure to solutions with high K^+ concentrations (> 100 mM) is considered to abolish active electrical responses by

clamping the cell membrane at a depolarized state. In a recent electrophysiological study on the RMA (Mulvany et al. 1982), exposure to a 125 mM K^+ (25 mM Na^+) solution (in the presence of phentolamine) was shown to reduce the membrane potential of the VSM cells to approximately -7 mV. It is reasonable to assume that addition of NA to a preparation bathing in such a K^+ -rich medium cannot cause a further depolarization of the cell membrane. Nevertheless, NA (10^{-5} M) further increased the active tension by approximately 70 % in chemically denervated arteries contracted by 124 mM K^+ (present study). Furthermore, arteries depolarized by K^+ and preincubated with a high concentration of nifedipine in order to reduce the K^+ -induced contraction to minimum, responded to NA with a sustained tonic contraction, which was only approximately 20 % smaller than that obtained in standard Krebs solution. A similar additive effect of NA on the response to K^+ was reported by Mulvany et al. (1982). In their study, addition of 0.8 μ M NA to the RMA previously contracted by 40 mM K^+ caused an approximately two-fold increase in the active tension, while the membrane potential depolarized by only about 3 mV. K^+ -induced tension development, on the other hand, was always accompanied by a reduction of the membrane potential (Mulvany et al. 1982). These results favour the view that the major part of the NA-induced contraction in the RMA is caused by pharmacomechanical coupling (Somlyo & Somlyo 1968).

Contribution of different Ca^{2+} pools

Based on the rate of tension increase, the contractions induced by both 124 mM K^+ and 10 μ M NA could usually be divided into an initial fast and an ensuing slow (tonic) component (cf. Bohr 1963, Watkins & Davidson 1980). The contractile response to K^+ in the RMA has previously been shown to be critically dependent on the presence of Ca^{2+} in the bath solution and, thus, seems to be caused mainly by influx of extracellular Ca^{2+} (Skärby et al. 1984). However, the RMA bathing in a Ca^{2+} -free medium responded to NA with a transient contraction, which was considered to reflect release of Ca^{2+} from a cellularly bound Ca^{2+} pool (Skärby et al. 1984). Since the tonic component of the response to NA was virtually abolished in Ca^{2+} -free medium, it was concluded that the activator Ca^{2+} mobilized during this part of the contraction is

derived mainly from the extracellular medium (Skärby et al. 1984). This conclusion is supported by the present study, showing that (1) removal of Ca^{2+} from the bath solution during the course of an established NA-induced contraction produced a rapid and nearly complete relaxation of the preparation and (2) 1 mM La^{3+} almost abolished the contractile response to NA. Furthermore, both K^{+} - and NA-induced contractions have been shown to be associated with an increased influx of $^{45}\text{Ca}^{2+}$ in the rat superior mesenteric artery, also supporting this contention (Godfraind & Dieu 1981, Godfraind & Miller 1982, Godfraind 1983).

Involvement of different Ca^{2+} entry pathways

Effect of nifedipine. Although the tonic component of the NA-induced contraction appeared to be almost entirely dependent of influx of extracellular Ca^{2+} , it was only slightly reduced by the Ca^{2+} -entry blocker nifedipine. This is in contrast to the strong relaxation produced by nifedipine of K^{+} -contracted arteries treated with prazosin. However, in those experiments where prazosin was not used to eliminate the adrenergic component, the relaxation produced by nifedipine developed considerably slower. Since nifedipine is known not to appreciably influence the process of adrenergic transmitter release (Starke & Schlömann 1973, Ebstein & Daly 1982, Högestätt et al. 1982, Larsson et al. 1984), stimulation of postjunctional α -adrenoceptors by endogenous NA released by K^{+} can probably explain this difference. The ability of nifedipine to differentiate between the tonic components of K^{+} - and NA-induced contractions, despite their similar dependency on extracellular Ca^{2+} , suggests that K^{+} and NA utilize partly different Ca^{2+} entry pathways to sustain contraction. This interpretation was further supported by studies of Ca^{2+} -induced contractions in Ca^{2+} -depleted arteries. In these experiments, nifedipine was found to be a considerably more effective inhibitor of Ca^{2+} -induced contractions in K^{+} -depolarized than in NA-exposed arteries. For example, 0.3 μM nifedipine completely abolished the contraction induced by 4.3 mM Ca^{2+} in K^{+} -depolarized arteries, whereas that in NA-exposed arteries was reduced by only approximately 40 %. In the perfused rat mesenteric vascular bed (Kondo et al. 1980) and in the isolated rat superior mesenteric artery (Godfraind 1983), nifedipine was

also found to preferentially counteract K^+ -induced contractions, as compared with those elicited by NA. This selective action of nifedipine on K^+ - over NA-induced responses was shared with diltiazem, but not with verapamil (Kondo et al. 1980). Since the concentration-response curves for the inhibitory effects of nifedipine on K^+ -induced contraction and $^{45}Ca^{2+}$ influx were almost superimposable, it was suggested that the action of nifedipine can be related to blockade of Ca^{2+} entry through channels opened during depolarization (Godfraind 1983).

Effect of La^{3+} . In contrast to nifedipine, La^{3+} failed to discriminate between K^+ - and NA-induced contractions. The fact that the ion was effective in μM concentrations are consistent with the idea that La^{3+} inhibits contraction by interacting with specific negatively charged membrane sites involved in Ca^{2+} translocation (van Breemen et al. 1972, Weiss 1974, Triggle 1981). Possibly, part of these sites may be identical with the negatively charge co-ordination sites suggested to be present at the external mouth of Ca^{2+} channels (Hurwitz 1978, Hagiwara and Byerly 1981, Kostyuk 1981).

Evidence of additivity. The Ca^{2+} sensitivity of the Ca^{2+} -depleted RMA was considerably higher after application of both K^+ and NA than in the presence of either stimulant separately. However, this observation does not implicate the existence of separate Ca^{2+} entry pathways in the muscle membrane, unless it can be shown that at least one of the stimulants produced a maximum effect on the membrane permeability to Ca^{2+} . Since the graded responses to Ca^{2+} in NA-exposed arteries were not significantly enhanced by increasing the NA concentration by a factor of ten, this criterion seems to be fulfilled. Moreover, the maximum contractile response to Ca^{2+} was approximately 40 % smaller in arteries depolarized by K^+ (in the presence of prazosin) than in those exposed to both K^+ and NA, providing further evidence of additivity. The presence of additivity indicates that the Ca^{2+} entry pathways utilized by K^+ and NA are at least partly independent. It also appears to rule out the possibility of a single pathway regulated by two separate mechanisms (cf. Meisheri et al. 1981).

Nature of the different Ca^{2+} entry pathways

Fig 11 schematically illustrates some possible Ca^{2+} entry routes, which may account for the Ca^{2+} influx during K^+ - and NA-induced contractions in the RMA.

POCs and ROCs. According to Bolton's (1979) definition of POCs and ROCs, the POC is an ion channel population that opens as the potential across the membrane is reduced, e.g. after K^+ depolarization. This channel is considered to correspond to the "slow channel" in heart muscle. Thus, in accordance with the slow channel, the POC has been shown to be effectively inhibited by Ca^{2+} -entry blockers (Bolton 1979, Nayler & Poole-Wilson 1981, Bou et al. 1983, Cauvin et al. 1983, Andersson & Högestätt 1984). ROCs, on the other hand, were suggested to be primarily controlled or operated by receptors for stimulant substances (Bolton 1979). The results of the present study are compatible with the view that K^+ (in the presence of prazosin) and NA promote the entry of Ca^{2+} by activating different populations of Ca^{2+} channel (POCs and ROCs), differing in their sensitivity to nifedipine (Fig 11). However, as will be discussed below, there are alternative explanations.

Na^+ - Ca^{2+} exchange carrier. It has been argued that a plasmalemma Na^+ - Ca^{2+} exchange carrier is involved in regulating the myoplasmic Ca^{2+} concentration in VSM (see van Breemen et al. 1979). Both an electroneutral (2 Na^+ for each Ca^{2+}) and an electrogenic (n Na^+ for each Ca^{2+} , $n \geq 3$) exchange have been implicated (see van Breemen et al. 1979). The presence of such a carrier mechanism would make the intracellular Ca^{2+} concentration sensitive to changes in both the Na^+ gradient over the cell membrane and the membrane potential if the exchange is electrogenic, or to the Na^+ gradient alone if the exchange process is electroneutral. Thus, the low Na^+ content and the depolarizing effect of the isotonic K^+ -rich solution used in the present study may theoretically cause contraction by suppressing or reversing a hypothetical Na^+ - Ca^{2+} extrusion mechanism. However, replacement of the NaCl in the standard Krebs solution with either sucrose, choline Cl or LiCl did not induce contraction in the RMA (E.D. Högestätt, unpublished observations). These results do not favour

the presence of a Na^+ - Ca^{2+} exchange carrier in the RMA and therefore speak against the involvement of such a mechanism in the K^+ -induced contraction in this preparation.

Alternative models for the receptor-operated pathway. Current information give us reason to consider two other models, which may also account for the nifedipine resistance of the Ca^{2+} entry pathway utilized by NA in the RMA and its apparent lack of dependence on the membrane potential. Both are based on an inability of nifedipine to influence the process of intracellular Ca^{2+} release and to prevent the refilling of the agonist-sensitive intracellular Ca^{2+} store (Fig 11b). Casteels and Droogmans (1981) suggested that filling of the NA-sensitive intracellular Ca^{2+} store in the rabbit ear artery occurs mainly through a direct pathway between the store and the extracellular medium. Unlike Mn^{2+} , D 600 was shown not to interfere with this pathway. In addition, neither D 600 nor nicardipine appreciably influenced the process of intracellular Ca^{2+} release. It was therefore suggested that a fast refilling of the intracellular Ca^{2+} store and subsequent release of Ca^{2+} from the store into the myoplasm could represent an alternative model for the ROC (Casteels & Droogmans 1981). Working with the rabbit aorta, Loutzenhiser and van Breemen (1983) argued that the Ca^{2+} which has just entered the myoplasm by, e.g., passive leak, rather than extracellular Ca^{2+} , serves as the immediate source of Ca^{2+} for the NA-sensitive intracellular Ca^{2+} store. In resting muscle, the Ca^{2+} buffering capacity of the store was assumed to prevent the passive leak of Ca^{2+} from causing muscle activation (Cauvin et al. 1983). It was further suggested that receptor occupation reduces the ability of the store to sequester Ca^{2+} (Loutzenhiser & van Breemen 1983). Consequently, following stimulation of the receptors, part of the Ca^{2+} entering the VSM cells by passive leak will no longer be prevented from reaching the contractile proteins. Considering that the passive leak of Ca^{2+} across the smooth muscle plasmalemma does not appear to be appreciably influenced by Ca^{2+} -entry blockers, this hypothesis may offer an explanation for the persistence of sustained agonist-induced contractions in the presence of Ca^{2+} -entry blockers, provided the passive leak of Ca^{2+} is large enough to cause muscle

contraction (see Cauvin et al 1983). In our view, this last model cannot completely account for the nifedipine resistance of the tonic component of the NA-induced contraction in the MA, since it would require an extremely high permeability of the cell membrane to Ca^{2+} under resting conditions that would have to be compensated for by intracellular Ca^{2+} uptake and/or binding and subsequent Ca^{2+} extrusion.

In conclusion, the results of the present study strongly suggest that K^+ and NA utilize partly different Ca^{2+} entry pathways to increase the myoplasmic Ca^{2+} concentration in the RMA. Whereas K^+ seems to promote the influx of Ca^{2+} by activation of Ca^{2+} channels sensitive to the membrane potential, the nature of the receptor-operated Ca^{2+} entry pathway remains to be established.

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Table 1. Ca^{2+} -induced contractions in rat mesenteric arteries exposed to either K^+ (in the presence or absence of $0.1 \mu\text{M}$ prazosin) or noradrenaline (NA), or to both stimulants in Ca^{2+} -free medium.

Stimulant	n	E_{max} (%) ^a	$\log \text{EC}_{50}$ ^b	Mean EC_{50} (M)
K^+ (124 mM)	6	109 ± 3	-3.72 ± 0.06	1.93×10^{-4}
K^+ (124 mM) + prazosin (10^{-7} M)	6	70 ± 3	-3.57 ± 0.09	2.69×10^{-4}
NA (10^{-5} M)	8	110 ± 5	-4.13 ± 0.07	7.35×10^{-5}
K^+ (124 mM) + NA (10^{-5} M)	6	111 ± 4	-4.54 ± 0.06	2.85×10^{-5}

^a Maximum contractile response expressed as per cent of the contraction evoked by $124 \text{ mM } \text{K}^+$ in standard Krebs solution ($1.5 \text{ mM } \text{Ca}^{2+}$).

^b Logarithm of the Ca^{2+} concentration producing half maximum contraction.

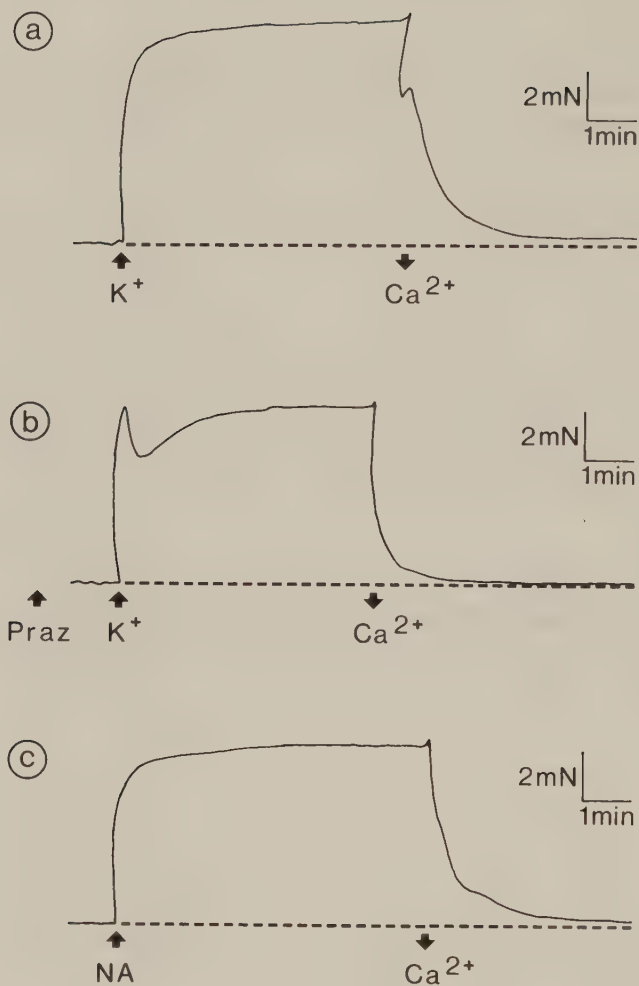


Fig 1. Reversal of established K^+ - and noradrenaline (NA)-induced contractions by Ca^{2+} withdrawal. (a) Contraction induced by a K^+ -rich solution, containing 124 mM K^+ . (b) Contraction evoked by K^+ in the continuous presence of 0.1 μ M prazosin (Praz). (c) Contraction elicited by 10 μ M NA. At downward arrows, the bath solution was changed to a Ca^{2+} -free medium (supplied with 1 mM EGTA), which otherwise contained the same constituents as the previous solution. Broken lines indicate the baseline tension.

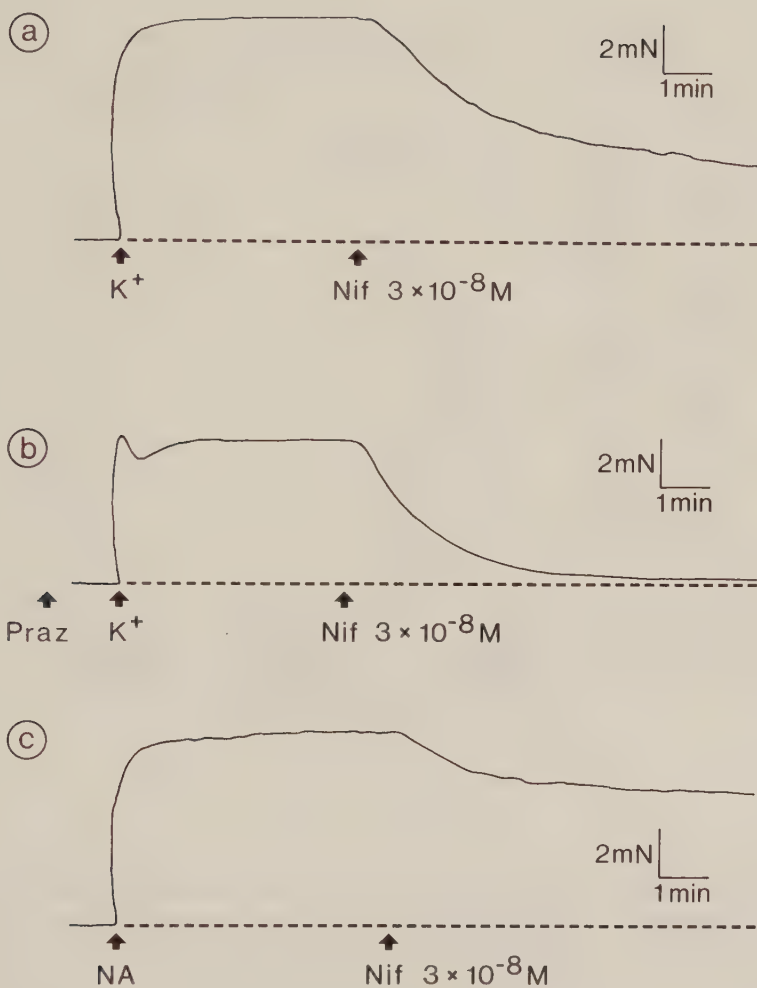


Fig 2. Relaxation of established K^+ - and noradrenaline-induced contractions by 30 nM nifedipine (Nif). For further explanations, see legend to Fig 1.

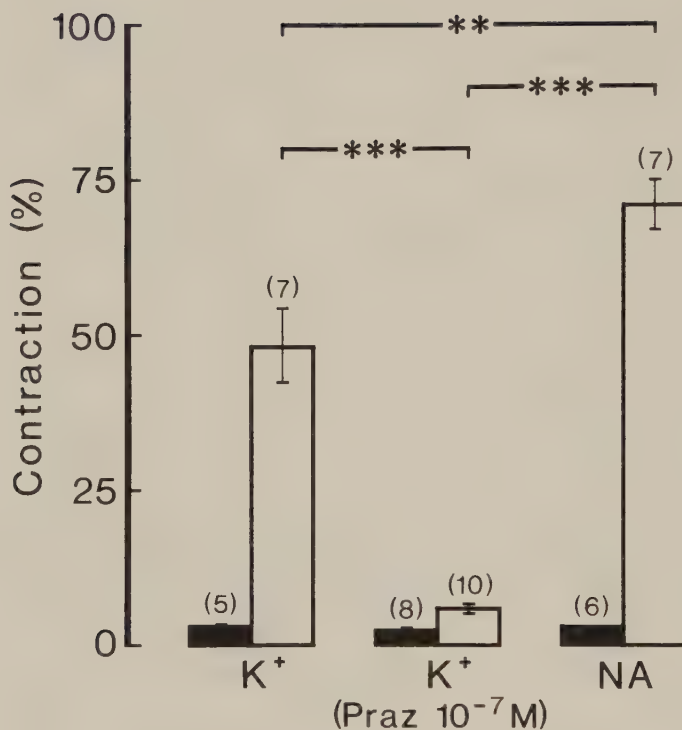


Fig 3. Effects of Ca^{2+} removal (filled columns) and 30 nM nifedipine (open columns) on rat mesenteric arteries contracted by 124 mM K^+ either in the absence or presence of 0.1 μ M prazosin (Praz), or by 10 μ M noradrenaline (NA). Each column refers to the amplitude of the contraction 5 min after Ca^{2+} withdrawal or application of nifedipine. The active tension recorded immediately prior to Ca^{2+} removal or nifedipine exposure was set to 100 %. Only preparations responding with stable contractions were exposed to Ca^{2+} -free medium or nifedipine. Figures within brackets indicate the number of experiments performed. Experimental conditions as in Fig 1 and 2. **, $P < 0.01$. ***, $P < 0.001$.

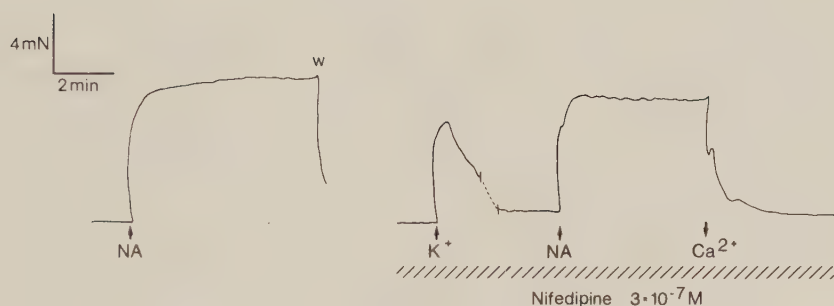


Fig 4. Contractile response to noradrenaline (10 μ M) in a K⁺-depolarized and nifedipine-exposed rat mesenteric artery, and its reversal by Ca²⁺ removal. From the left to the right: **First upward arrow**, control contraction elicited by noradrenaline (NA) in standard Krebs solution. **Second upward arrow**, exposure to 124 mM K⁺ in the presence of 0.3 μ M nifedipine. Nifedipine was added 20 min before K⁺ and then kept present throughout the experiment. **Third upward arrow**, addition of NA to the K⁺-depolarized artery. At **downward arrow**, the bath solution was changed to a Ca²⁺-free, K⁺-rich medium, which apart from NA and nifedipine also contained 1 mM EGTA. Note the large transient response to K⁺ caused by neurally released NA, as it was almost abolished by phentolamine (1 μ M) or prazosin (0.1 μ M). Broken line indicates a time lapse of approximately 5 min. W=wash with standard Krebs solution.

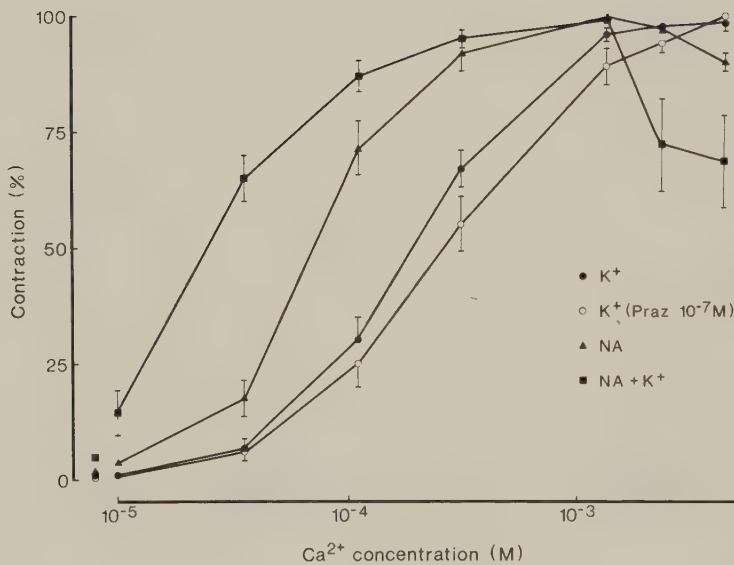


Fig 5. Concentration-response relationship for Ca^{2+} in rat mesenteric arteries exposed to: (1) 124 mM K^+ ($\bullet$, $n=6$), (2) 124 mM K^+ in the presence of 0.1 μM prazosin (Praz) ($\circ$, $n=6$), (3) 10 μM noradrenaline (NA) ($\blacktriangle$, $n=8$), or (4) 124 mM K^+ and 10 μM NA ($\blacksquare$, $n=6$). The maximum contractile response induced by Ca^{2+} was set to 100 % in all experiment. Each vessel segment was exposed to only one cumulative application of Ca^{2+} . The symbols in the left-hand bottom corner indicate the active tension immediately prior to addition of Ca^{2+} . For further explanations, see "Materials and Methods".

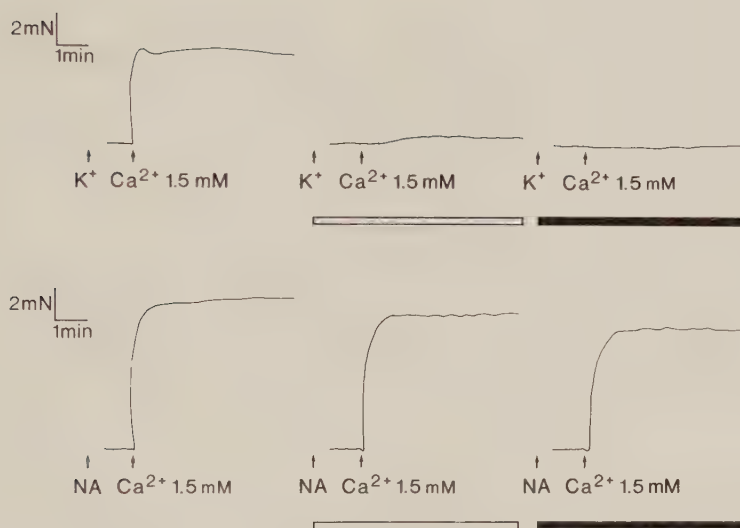


Fig 6. Effects of 30 nM (open bars) and $0.3 \mu\text{M}$ (filled bars) nifedipine on Ca^{2+} -induced contractions in rat mesenteric arteries exposed either to 124 mM K^+ in the presence of $0.1 \mu\text{M}$ prazosin (upper panel) or to $10 \mu\text{M}$ NA (lower panel) in Ca^{2+} -free medium. The time of exposure to each nifedipine concentration before addition of Ca^{2+} was approximately 20 min . For further explanations, see "Materials and Methods".

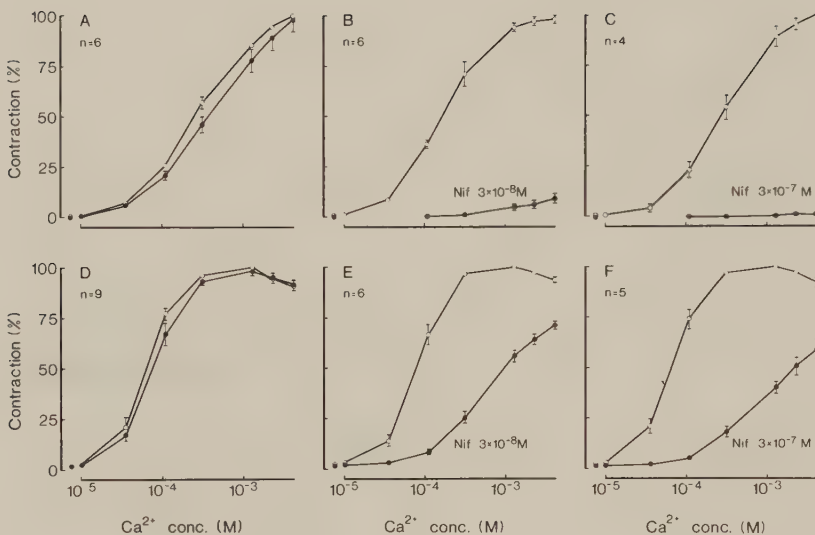


Fig 7. Effects of nifedipine (Nif) on the concentration-response relationship for Ca^{2+} in K^+ (124 mM)-depolarized (upper panel) and noradrenaline (0.1 μM)-exposed (lower panel) rat mesenteric arteries. Two cumulative applications of Ca^{2+} were performed in each arterial segment, the second obtained either in the absence (controls, A and D) or in the presence of 30 nM (B and E) or 0.3 μM (C and F) nifedipine. Open symbols refer to the first and filled symbols to the second application of Ca^{2+} . 100 % denotes the maximum contractile response obtained on the first application of Ca^{2+} . The time of exposure to nifedipine before addition of Ca^{2+} was approximately 20 min. The symbols in the left-hand bottom corner in each diagram indicate the active tension immediately before addition of Ca^{2+} . For further explanations, see "Materials and Methods".

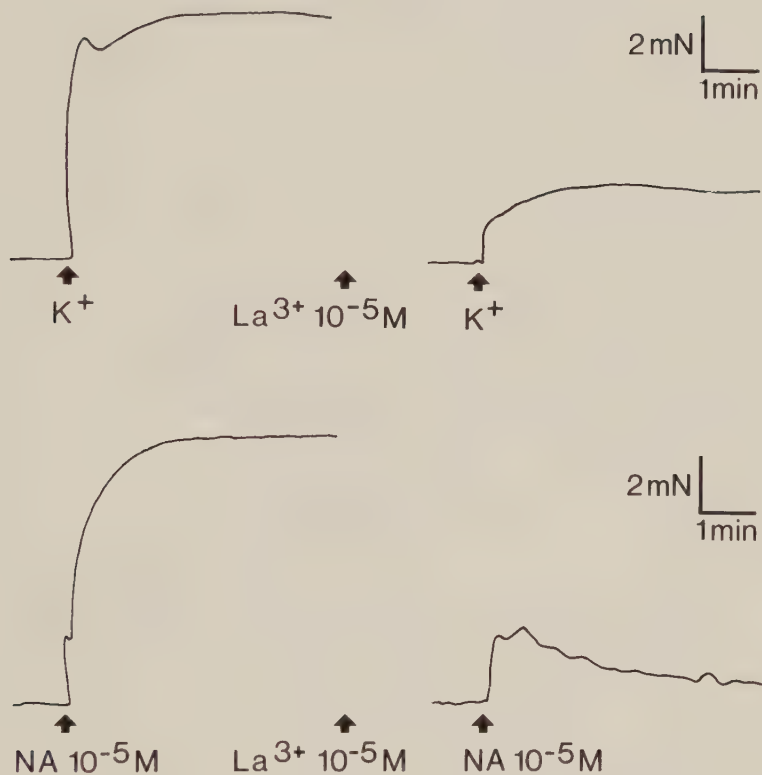


Fig 8. Effects of $10 \mu M La^{3+}$ on the contractile responses to $124 mM K^+$ and $10 \mu M$ noradrenaline (NA). Responses to K^+ were obtained in the continuous presence of $0.1 \mu M$ prazosin.

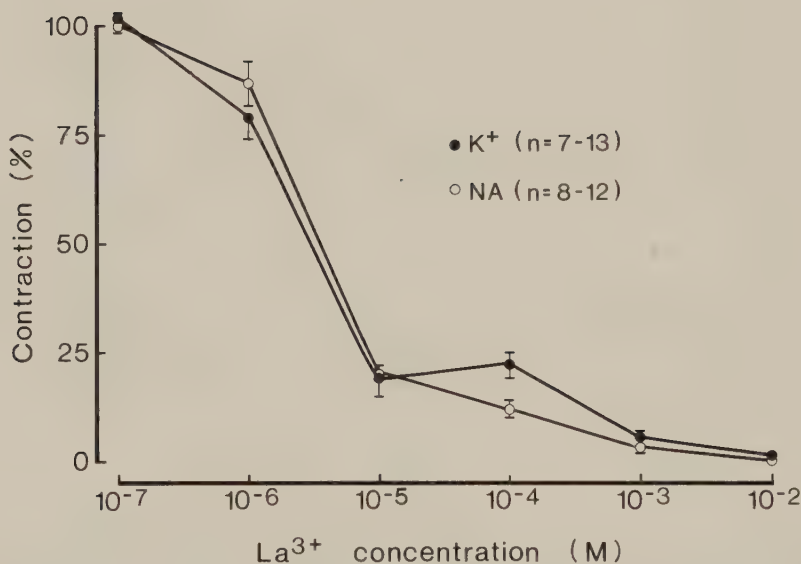
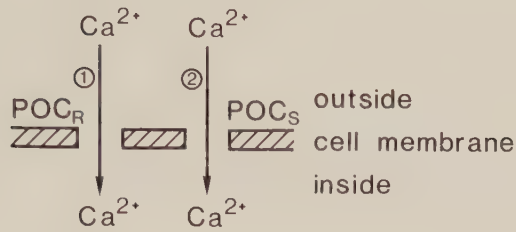


Fig 9. Inhibition of K⁺ (124 mM)- and noradrenaline (NA, 10 μ M)-induced contractions by the indicated concentrations of La³⁺. The maximum response obtained during the first 5 min of exposure to the stimulants was used for quantifying the inhibition produced by La³⁺. The mean amplitude of two control contractions elicited by K⁺ or NA immediately prior to La³⁺ application was set to 100 %. Each preparation was exposed to only one La³⁺ concentration. Prazosin (0.1 μ M) was present in the bath solution in all experiments with K⁺.

(a)

Nifedipine sensitive

(b)

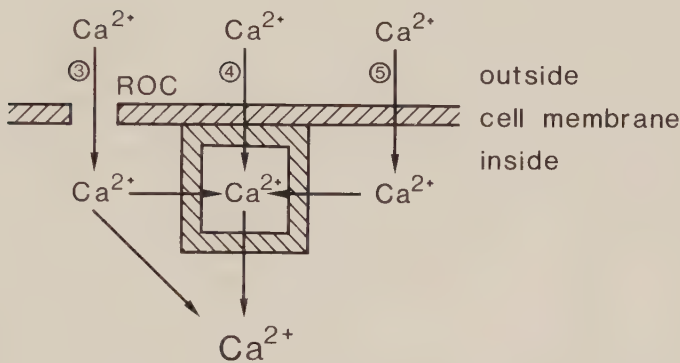
Nifedipine insensitive

Fig 10. (a) Possible Ca^{2+} entry routes activated by K^+ depolarization and sensitive to nifedipine: (1) Ca^{2+} influx via a rapidly activated, potential-operated channel (POC_R), responsible for the fast component of the contractile response to K^+ . (2) Ca^{2+} influx via a slowly activated, potential-operated channel (POC_S), responsible for the slow component of the K^+ -induced contraction. (b) Possible Ca^{2+} entry routes utilized by noradrenaline (NA) and relatively resistant to nifedipine: (3) Ca^{2+} influx via a receptor-operated channel (ROC). (4) Refilling of the NA sensitive intracellular Ca^{2+} store by a direct pathway from the extracellular medium and subsequent release of Ca^{2+} from the store into the myoplasm. (5) "Passive leak" of Ca^{2+} across the cell membrane. Immediately upon entering the myoplasm, this Ca^{2+} is actively pumped into the NA sensitive intracellular Ca^{2+} store, thus participating in the refilling of the store, from where it is subsequently released to activate the contractile proteins.



**ON THE POSTJUNCTIONAL α -ADRENOCEPTORS
IN RAT CEREBRAL AND MESENTERIC ARTERIES**

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Running title: Postjunctional α -adrenoceptors in rat
arteries

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ABSTRACT

1. The contractile response to exogenously applied nor-adrenaline (NA) was examined in vitro in tubal segments (0.2-0.1 mm in diameter) of rat middle cerebral (MCA), basilar (BA) and mesenteric (MA) arteries.

2. In the MCA, the maximum contractile response to NA (10^{-4} M) was considerably smaller than that induced by K^+ (124 mM) or 5-hydroxytryptamine (10^{-5} M), whereas the inverse relationship was found in the MA. NA usually failed to elicit contraction in the BA even in the presence of propranolol and cocaine.

3. Propranolol (3×10^{-7} M) enhanced the maximum contractile response to NA by approximately 100 % in the MCA without affecting the potency of the agonist. In the MA, propranolol had no effect on the concentration-response relationship for NA. Cocaine (10^{-5} M) or 6-hydroxydopamine pretreatment increased the NA sensitivity of the MA by a factor of three, whereas these procedures failed to influence the NA sensitivity of the MCA.

4. A marked stereoselectivity was found in the MCA, as (-)-NA was more than 100 times more potent than (+)-NA as a contractile agent. The order of potency of a series of α -adrenoceptor agonists was (-)-adrenaline > oxymetazoline > ($\pm$)-NA $\approx$ (-)-phenylephrine > methoxamine in the MCA and ($\pm$)-NA > (-)-phenylephrine in the MA. Clonidine failed to elicit contraction in concentrations lower than 3×10^{-4} M in both types of artery.

5. Prazosin was between three and four orders of magnitude more potent than rauwolscine in inhibiting NA-induced contractions in the MCA and MA. The pA_2 values for, respectively, prazosin and rauwolscine were 9.3 and 5.4 in the MCA and 9.7 and 6.8 in the MA. The slope of the Schild plot deviated significantly from unity only for rauwolscine in the MA (0.64).

6. It is concluded that the contractile response to exogenous NA in the MCA and MA is mediated mainly by stimulation of α_1 -adrenoceptors, although the involvement of a small population of postjunctional α_2 -adrenoceptors in the MA cannot be excluded. In contrast to the MCA, the BA appears to lack contraction-mediating α -adrenoceptors, indicating regional differences in the α -adrenoceptor distribution in the rat cerebrovascular bed.

Key words. Mesenteric arteries; cerebral arteries; rat; α -adrenoceptor subtypes; β -adrenoceptors

INTRODUCTION

The pithed rat is a commonly used experimental model for studies of drugs influencing blood pressure by action on, e.g., α -adrenoceptors. However, details on the interaction between drugs and α -adrenoceptor sites may be difficult to analyze in this model. Therefore, studies of isolated blood vessels under well controlled in vitro conditions are desirable. There is ample evidence to support the view that both α_1 - and α_2 -adrenoceptors occur postjunctionally in rat vascular smooth muscle (VSM); stimulation of either type of receptor leads to vasoconstriction (Timmermans et al. 1979, Docherty & McGrath 1980, Kobinger & Pichler 1980, Gerold & Heusler 1983). However, the regional distribution of these receptors in the rat vascular system has not been established.

It is well known that the pithed rat reacts to selective α_2 -adrenoceptor stimulation with a marked increase in blood pressure (Timmermans et al. 1979, Docherty & McGrath 1980, Kobinger & Pichler 1980, Gerold & Heusler 1983). Since the resistance vessels in the mesenteric circulation presumably have importance for blood pressure regulation (Mellander & Johansson 1968), a postjunctional α_2 -adrenoceptor population of functional importance would be anticipated in this region. However, binding studies have yielded conflicting data. Colucci et al. (1980, 1981) reported that the α -adrenoceptors in rat mesenteric arteries were predominantly of the α_1 -type, whereas Dashwood (1983), using autoradiographic technique, failed to demonstrate any specific binding sites for prazosin in this tissue. Therefore, it would be of interest to functionally characterize the α -adrenoceptors in this vascular region.

Previous studies have suggested the existence of mainly α_2 -adrenoceptors in cerebral arteries from cat (Medgett & Langer 1983, Skärby et al. 1983), dog (Sakakibara et al. 1982, Toda 1983) and cattle (Tsukahara et al. 1983). However, the post-junctional α -adrenoceptors in rat cerebrovascular smooth muscle have not been characterized. As the rat is extensively used in studies of the cerebral circulation, there is a need of further information on the α -adrenoceptor function in rat cerebral vessels.

In the present study, rat arteries from the mesenteric and cerebral circulations were selected for investigation of α -adrenoceptor subtypes by means of selective agonists and antagonists. A method allowing study of isolated blood vessels with a diameter down to 0.1 mm was used (Högestätt et al. 1983).

MATERIALS AND METHODS

Vascular preparations

Cerebral and mesenteric arteries were obtained from adult Sprague-Dawley rats of either sex, weighing between 200 and 300 g. The animals were killed by decapitation, and the middle cerebral artery (MCA, diameter ≈ 0.1 mm), the basilar artery (BA, diameter ≈ 0.2 mm) and segments of the second generation of branches originating from the superior mesenteric artery (MA, diameter ≈ 0.2 mm) dissected out. The excised arteries were either used immediately for experimentation or stored in "standard" Krebs solution at 8 to 12 °C for no longer than 24 hours. The standard Krebs solution had the following composition (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, (+)-glucose 6.0.

6-hydroxydopamine treatment

In order to destroy peripheral adrenergic nerve endings, 100 mg/kg of 6-hydroxydopamine (6-OHDA) was injected into a tail vein 24 hours before sacrifice. 6-OHDA was dissolved in ice cold 1 % ascorbic acid, giving a final concentration of 100 mg/ml. The solution with 6-OHDA was kept on ice and protected from light until injection.

Recording of mechanical activity

The experimental equipment and the procedure used for recording of mechanical activity have been described previously (Högestätt et al. 1983). Briefly, ring segments of the arteries, approximately 1 to 2 mm long, were suspended between two stainless steel wires (diameter = 50 - 100 μm) in a temperature-controlled (37°C) small volume muscle bath (2.5 ml), containing standard Krebs solution. The bath solution was continuously bubbled with 95 % O_2 and 5 % CO_2 , resulting in a pH of approximately 7.4. Mechanical activity was recorded "isometrically" by means of a Grass Instruments FT03C force-displacement transducer, connected to one of the two steel wires. During an equilibration period of approximately 60 min, the vascular preparation was stretched on repeated occasions until a stable resting tension of approximately 1.5 mN (MCA) or 2 mN (BA, MA) was obtained.

Experimental procedure

After the accommodation period, the contractile capacity was assayed by exposing the arterial segment to an isotonic 124 mM K^+ solution (prepared by substituting all NaCl in the standard Krebs solution with an equimolar amount of KCl). The arteries were then subjected to repeated single (10^{-5} M) or cumulative applications of NA with approximately 30 min intervals. Preparations that failed to produce reproducible responses to NA were discarded. Contractions were considered reproducible when the maximum tensions of two consecutive contractions differed by less than 10 %.

Each arterial segment was usually exposed to only one agonist (added cumulatively) besides NA. However, on rare occasions when the effects of more than one agonist were tested, NA was added in between the agonists to ensure that the artery had recovered from the previous agonist exposure.

The effects of propranolol, cocaine and the different α -adrenoceptor blockers on the concentration-response relationship for NA were examined according to the following protocol. The concentration-response relationship for NA was first determined under control conditions. After thorough washout of the agonist, the arteries were incubated with the different drugs for approximately 20 min and the concentration-response relationship for NA was subsequently determined in the presence of the drugs. This sequence was then repeated with increasing concentrations of the drugs. When the effects of the different α -adrenoceptor agonists and antagonists were compared in the MCA and MA, 3×10^{-7} M propranolol (MCA) or 3×10^{-7} M propranolol and 10^{-5} M cocaine (MA) were always present in the bath solution.

Unlike the MA, the MCA tended to develop spontaneous contractile activity after repeated applications of NA. Such contractions were shown to considerably alter the concentration-response relationship for NA. Moreover, the contractile response to 10^{-3} M NA in the presence of α -adrenoceptor blockers frequently required up to 20 min to reach a maximum in the MCA. This preparation was therefore usually exposed to only one concentration of the different drugs to shorten the length of each experiment. The MA, on the other hand, was exposed to

between one and five consecutive concentrations of the drugs. As shown in control arteries, the contractile response to NA remained reproducible throughout the experiments using this protocol.

When NA-induced relaxation was studied in cerebral arteries, the vessels were first contracted by 2.5×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a concentration producing approximately 90 % of the maximum response to the agonist. NA was only added to arteries responding to $PGF_{2\alpha}$ with stable contractions.

Calculations and statistical analysis

The maximum contractile response induced by each concentration of agonist was used for construction of concentration-response curves. The agonist concentration producing 50 % of its own maximum contraction (EC_{50}) was determined graphically for each curve by linear interpolation. The $\log EC_{50}$ values were subsequently used for calculation of geometric mean values. $E_{\max}$ was defined as the maximum contraction attainable by the agonist.

pA_2 values for prazosin and rauwolscine were determined according to the method of Arunlakshana and Schild (1959) (Schild plot), using NA as the agonist. Briefly, the $\log$ (concentration ratio (CR)-1) was plotted against the $\log$ antagonist concentration (M), where CR is the ratio of NA concentrations giving equal responses in the absence and presence of antagonist. The CR was determined for contractions corresponding to 50 % of the maximum response to NA before application of the α -adrenoceptor blockers. If the antagonism is competitive, the Schild plot should yield a straight line with a slope of 1 and an intercept along the abscissa (pA_2 value) equal to the $-\log$ dissociation constant of the antagonist ($-\log K_B$) (Arunlakshana & Schild 1959, Kenakin 1982).

Calculation of linear regressions for the Schild plots was performed on a Hewlett Packard 41CV minicalculator, using the least squares method. The program used was kindly supplied by Dr Claus Rerup, Department of Pharmacology, University of Lund, Lund, Sweden. The results shown in tables, figures and the text are

expressed as mean values $\pm$ SEM (unless specified otherwise); n denotes the number of arteries examined. Statistical analysis of the results was performed by using Student's t-test (two-tailed) for unpaired or paired data when applicable. Statistical significance was accepted when $P < 0.05$.

Drugs

The drugs used were ($\pm$)-noradrenaline HCl, (-)-adrenaline bitartrate, 5-hydroxytryptamine creatinine sulfate, dopamine HCl, (-)-phenylephrine HCl, yohimbine HCl, 6-hydroxydopamine HBr (Sigma), clonidine HCl (Boehringer Ingelheim), cocaine HCl (ACO), (-)-noradrenaline HCl (Aldrich), (+)-noradrenaline HCl (Hoechst), isoprenaline HCl (Riker), methoxamine HCl (Burroughs Wellcome) oxymetazoline HCl (Draco), phentolamine methanesulfonate (Ciba-Geigy), phenoxybenzamine HCl (Smith, Kline and French), prazosin HCl (Pfizer), rauwolscine HCl (Roth) and ($\pm$)-propranolol HCl (ICI). Unless specified otherwise, the ($\pm$)-form of NA was always used.

All drugs except prazosin and 6-OHDA were dissolved in 0.9 % NaCl, containing 1 mM ascorbic acid. Prazosin was dissolved in 10 % lactic acid or 1 mM HCl, giving a final concentration of approximately 10^{-3} M, and further diluted with water. Because of the difficulty to dissolve prazosin in HCl, a few drops of methanol were first added to the dry substance. The solvents used did not significantly affect the contractile response to NA in the MCA and MA. Stock solutions of the drugs were stored at -70°C and fresh dilutions were made daily. Prostaglandin $\text{F}_{2\alpha}$ (dissolved in water) was provided in ampoules (Astra). The chemicals used were of analytical grade and redistilled water was used for preparing all solutions. All drugs were added directly into the muscle bath in volumes of 5 to 25 μl and the concentrations reported are the calculated final concentrations in the bath solution.

RESULTS

Contractile response to NA

NA induced concentration-dependent contractions in the MCA and MA, whereas the agonist produced only a minimal or no response in the BA (Fig 1 and 2). The EC_{50} values for NA in the MCA and MA are given in Table 1. When expressed as per cent of the contractile response to 124 mM K^+ , the maximum contraction evoked by NA was considerably smaller in the MCA ($31 \pm 5 \%$, $n=6$) than in the MA ($111 \pm 2 \%$, $n=6$), although that elicited by 5-hydroxytryptamine (10^{-5} M) did not differ significantly between the MCA ($86 \pm 4 \%$, $n=8$) and the MA ($85 \pm 4 \%$, $n=8$). Figure 2 gives a comparison between the contractile responses to 124 mM K^+ and 10^{-5} M NA in the MCA, BA and MA. As can be seen, exposure of the MCA to 10^{-5} M NA induced a rapid contraction, which after an initial peak declined to a lower tension level (tonic component). A similar "escape" from contraction, although less pronounced, was frequently observed during cumulative applications of NA. In contrast, this phenomenon was never seen in the MA.

Effects of propranolol, cocaine and 6-OHDA pretreatment

Figure 3 shows the contractile response to 10^{-5} M NA in an MCA before and after application of $3 \times 10^{-7} \text{ M}$ propranolol. Propranolol increased the tonic component of the NA-induced contraction by $136 \pm 17 \%$ ($n=6$) and, hence, prevented or substantially reduced the escape from contraction. The effects of propranolol and cocaine on the NA concentration-response relationship in the MCA and MA are shown in figure 4 (see also Table 1). Propranolol ($3 \times 10^{-7} \text{ M}$) significantly increased the maximum NA-induced contraction in the MCA by $113 \pm 27 \%$ ($n=9$) without altering the NA sensitivity of the artery; exposure to 10^{-6} M propranolol did not further increase the response to NA. In the MA, propranolol ($3 \times 10^{-7} - 10^{-6} \text{ M}$) failed to affect the concentration-response relationship for NA. Cocaine ($10^{-6} - 10^{-4} \text{ M}$), on the other hand, significantly increased the NA sensitivity of the MA without appreciably influencing the maximum response; a maximum effect was produced by 10^{-5} M cocaine, which increased the NA sensitivity of the MA by a factor of three (Table 1). In contrast, in the MCA, cocaine (10^{-6} M) reduced the maximum contractile response to NA by approximately 10 % without

significantly affecting the EC_{50} value for the agonist. The drug was therefore excluded from the bath solution in all subsequent experiments on this preparation. In the BA, application of a single high concentration of NA ($> 10^{-5}$ M) in the presence of 3×10^{-7} M propranolol and 10^{-6} M cocaine occasionally induced a transient contraction, less than 5 % of the contractile response to 124 mM K^+ .

In agreement with the experiments with cocaine, MAs obtained from 6-OHDA treated rats were approximately three times more sensitive to NA than were those from untreated rats, whereas MCAs from the two groups of rat were equally sensitivity to NA (Table 1). In addition, the contractile response to NA in 6-OHDA pretreated MAs was not affected by 10^{-5} M cocaine ($n=3$), confirming that the chemical denervation had been successful.

Although the escape from contraction observed in the MCA was considerably reduced by 3×10^{-7} M propranolol (see above), it was still frequently observed after exposures to 10^{-4} and 10^{-3} M NA. In order to test whether there was a breakthrough of the propranolol blockade at high NA concentrations, the following experiments were carried out. The MCA (exposed to 10^{-6} M prazosin) and BA were contracted by 2.5×10^{-6} M $PGF_{2\alpha}$, and the relaxation induced by NA was determined before and after application of 3×10^{-7} M propranolol. Figure 5 shows the results from such an experiment. It is evident that 3×10^{-7} M propranolol was not sufficient to abolish the relaxation produced by NA concentrations higher than 10^{-5} M.

Effects of adrenoceptor agonists

The contractile effects of ($\pm$)-NA, (-)-NA, (+)-NA, (-)-adrenaline, isoprenaline and dopamine in the MCA are shown in figure 6a. A marked stereoselectivity was found, since (-)-NA was more than 100 times as potent as (+)-NA, and (-)-NA was approximately twice as potent as ($\pm$)-NA (Table 2). Adrenaline was significantly more potent than NA (Table 2), whereas isoprenaline failed to contract the MCA. Dopamine was a weak agonist.

The MCA was contracted by α -adrenoceptor agonists in the order of potency oxymetazoline $>$ (-)-phenylephrine $>$ methoxamine (Fig 6

b, Table 2). Clonidine failed to elicit contraction in concentrations lower than 3×10^{-4} M (Fig 6b). Methoxamine was a full agonist when compared with NA, and the maximum contractile response to phenylephrine was only slightly smaller than that produced by NA (Table 2). Contractions induced by oxymetazoline developed considerably slower than those elicited by the other α -adrenoceptor agonists, and the effect usually persisted after frequent washes in agonist-free medium. Oxymetazoline behaved as a partial agonist, the maximum contraction being approximately 40 % smaller than that produced by NA (Table 2). Moreover, the contractile response to NA was usually reduced after a previous exposure to oxymetazoline. Notably, oxymetazoline was also found to induce concentration-related and sustained contractions in the BA, whereas the other α -adrenoceptor agonists failed to do so.

In the MA, (-)-phenylephrine induced concentration-dependent contractions (Fig 7); the EC_{50} value was 2.1×10^{-6} M ($-\log EC_{50} = 5.67 \pm 0.09$, $n=9$) and the maximum response was only about 5 % smaller than that evoked by ($\pm$)-NA. Clonidine, on the other hand, failed to elicit contraction (Fig 7).

Effects of α -adrenoceptor antagonists

The classical α -adrenoceptor antagonists phentolamine (10^{-6} M) and phenoxybenzamine (10^{-6} M, 20 min pretreatment) abolished (phenoxybenzamine) or substantially reduced (phentolamine) the contractile response to 10^{-5} M NA in the MCA and MA.

In the MCA, prazosin effectively inhibited the NA-induced contractions and a significant effect was observed at concentrations as low as of 10^{-10} M. As can be seen in figure 8a, the inhibition produced by prazosin was surmountable, although the concentration-response curves for NA in the presence of 10^{-9} to 10^{-7} M prazosin did not reach a clear plateau. Construction of a Schild plot for prazosin gave a regression line with a slope close to unity and a pA_2 value of approximately 9.3 (Fig 9, Table 3). Rauwolscine and yohimbine were considerably less effective inhibitors of NA-induced contractions in the MCA than was prazosin (Fig 8 b, c). Notably, 10^{-6} M yohimbine significantly increased the maximum contractile response to NA by approximately 20 %. The regression line of the Schild plot for

rauwolscine had a slope of about 1.4, and the pA_2 value was estimated as approximately 5.4 (Fig 9, Table 3).

In the MA, prazosin shifted the concentration-response curve for NA to the right without appreciably influencing the maximum response or the slope (Fig 10a). A similar displacement was produced by rauwolscine, although the drug was considerably less potent than prazosin (Fig 10b). Schild plots for the antagonism of NA-induced contractions by prazosin and rauwolscine are shown in figure 11. The slope of the Schild plot was close to unity for prazosin, whereas that for rauwolscine was significantly lower than unity. The pA_2 values were determined to approximately 9.7 for prazosin and 6.8 for rauwolscine. The results from the Schild plots are summarized in table 3.

Concentration-response curves for the inhibitory effects of prazosin and rauwolscine in the MCA and MA (relating the contraction induced by 10^{-5} M NA to the antagonist concentration) are shown in figure 12. The degree of inhibition was calculated from the same experiments as those presented in figure 7 and 9 by expressing the amplitude of the contractile response to 10^{-5} M NA in the presence of prazosin or rauwolscine as per cent of that elicited by the same concentration of NA before application of the α -adrenoceptor blockers. It is evident that prazosin was between three and four orders of magnitude more potent than rauwolscine in inhibiting the NA-induced contractions in the MCA and MA. Similar results were obtained when the inhibition produced by the α -adrenoceptor blockers was calculated for 10^{-6} and 10^{-4} M NA (not shown). The effects of prazosin, rauwolscine and yohimbine were reversible, as frequent washes in antagonist-free solution restored the response to NA. Neither of the α -adrenoceptor blockers influenced the resting tension of the arteries.

DISCUSSION

Perivascular adrenergic nerves have been demonstrated in the major cerebral arteries in several mammals, including rat (see Edvinsson & MacKenzie 1977). Studies of isolated rabbit, dog and cat cerebral arteries preincubated with ^3H -NA have indicated that the adrenergic nerves accumulate the labelled transmitter and release it upon electrical field stimulation (Duckles 1980, Sakakibara et al. 1981, Skärby 1984). However, the physiological role of the sympathetic nerves have been a matter of considerable debate (Edvinsson & MacKenzie 1977, Heistad & Marcus 1978, Purves 1978, McCalden 1981). Despite the existence of functioning nerves, a large bulk of information from several animal species and man support the view that NA is a considerably weaker contractile agent in cerebral arteries than in peripheral arteries (Toda & Fujita 1973, Müller-Schweinitzer & Weidmann 1977, Rose & Moulds 1979, Högestätt et al. 1982, Skärby et al. 1983). As demonstrated in the present study, this seems to apply also to the rat.

The contractile response to NA in the rat MCA and MA was effectively inhibited by the classical α -adrenoceptor blocking agents phentolamine and phenoxybenzamine, indicating that the NA-induced contractions were caused by stimulation of α -adrenoceptors. The observation that (-)-adrenaline was more potent than (-)-NA in the MCA, whereas isoprenaline failed to elicit contraction, further supported this view. In this respect the rat MCA appeared to differ from the cat MCA, in which isoprenaline was found to be a full agonist (Edvinsson & Owman 1974). The weak effect of dopamine in the rat MCA does not favour the existence of specific contraction-mediating dopamine receptors in this preparation, but rather suggests that the contractile response to this agonist was caused by activation of α -adrenoceptors.

Propranolol increased the maximum contractile response to NA in the rat MCA by approximately 100 %, but had no effect on the NA concentration-response relationship in the rat MA. This suggests that stimulation of postjunctional β -adrenoceptors functionally opposed the α -adrenoceptor-mediated contractile effect of NA in the MCA, but not in the MA. A similar β -adrenoceptor-mediated modulation has been suggested to occur in

the cat MCA (Medgett & Langer 1983). Dog (Toda 1974) and goat (Urquilla et al. 1975) cerebral arteries, on the other hand, appear to be only sparsely supplied with relaxing β -adrenoceptors. The failure of propranolol to enhance NA-induced contractions in the rat MA does not exclude the presence of β -adrenoceptors in this preparation, since any β -adrenoceptor-mediated effect might have been masked by the strong contractile response to NA. In fact, Asano et al. (1982) reported that although propranolol failed to influence the concentration-response relationship for NA in the isolated rat superior MA, phenoxybenzamine-treated preparations contracted by K^+ responded to NA and isoprenaline with a concentration-dependent relaxation.

Even in the presence of propranolol, the E_{max} for NA was approximately 60 % smaller (relative to the maximum contraction induced by K^+ or 5-HT) in the rat MCA than in the rat MA. The low responsiveness of the MCA to NA cannot be attributed to removal of NA from the biophase by an avid neuronal uptake mechanism, since cocaine failed to augment the NA-induced contractions. This seems to be a general feature of cerebrovascular smooth muscle, since the contractile response to NA in cerebral arteries from rabbit, cat or dog was not significantly potentiated by cocaine or sympathectomy (Duckles & Bevan 1976, Toda et al. 1978, Araki et al. 1982). However, in the rat MA, cocaine increased the sensitivity to NA by a factor of three. Similarly, MAs obtained from 6-OHDA treated rats were approximately three times more sensitive to NA than were those from untreated rats. However, MCAs removed from the two groups of rats did not differ significantly in their sensitivity to NA. Nonetheless, both vascular regions are known to receive an abundant supply of adrenergic nerves (Furness 1973, Kobayashi et al. 1981). This suggests differences between the arteries in the function of the adrenergic neuroeffector apparatus, possibly related to a larger neuromuscular distance in cerebral than in peripheral arteries, as observed by Lee et al. (1980) in the rabbit. A close neuromuscular distance has been suggested to be critical for cocaine-induced prejunctional supersensitivity (Verity 1969).

When the influence of the neuronal uptake mechanism had been reduced by cocaine or 6-OHDA pretreatment, NA was approximately

2.5 times more potent in the rat MA than in the rat MCA. The lower potency and the smaller intrinsic activity of NA in the MCA, as compared with the MA, may reflect differences between the arteries in the α -adrenoceptor density and/or in the coupling between the receptors and the cellular response.

Exposure of the rat MCA to NA in the absence of propranolol usually induced an initial rapid contraction followed by a tonic response of lower amplitude. A similar escape from contraction has previously been observed in isolated rabbit basilar and femoral arteries, and cat mesenteric arteries after application of NA (Ross 1975, Duckles & Bevan 1976, Asano et al. 1982). In the rat MCA, propranolol significantly increased the tonic component of the NA-induced contraction, suggesting that stimulation of inhibitory β -adrenoceptors was partly responsible for this phenomenon. However, in rabbit basilar and cat mesenteric arteries, propranolol failed to influence the contractile response to NA, whereas in rat femoral arteries, propranolol prevented the escape from contraction (Ross 1975, Duckles & Bevan 1976, Asano et al. 1982), suggesting that there can be more than one mechanism behind this phenomenon.

In the present study, propranolol (3×10^{-7} M) was always included in the bath solution when the effects of the α -adrenoceptor blockers on the concentration-response relationship for NA were examined. Interestingly, this concentration of propranolol was not sufficient to abolish the relaxation produced by NA in the PGF₂ α -contracted rat MCA (in the presence of prazosin) and BA, and a breakthrough of the propranolol blockade was usually observed after application of NA concentrations higher than 10^{-5} M. This observation may be of methodological interest, since most in vitro studies concerned with the classification of α -adrenoceptors in VSM have used propranolol concentrations which may not be expected to fully abolish β -adrenoceptor-mediated effects of NA.

Studies on pithed rats have indicated the existence of both α_1 - and α_2 -adrenoceptors postjunctionally in VSM (Timmermans et al. 1979, Docherty & McGrath 1980, Kobinger & Pichler 1980, Gerold & Heusler 1983). However, the distribution of these receptors in

the rat vascular system has not been established. The present in vitro study suggests that the contractile response to NA in the rat MCA and MA is mediated by stimulation of predominantly α_1 -adrenoceptors. This conclusion is based on the following observations. The selective α_2 -adrenoceptor agonist clonidine failed to elicit contraction in concentrations lower than 3×10^{-4} M in both types of artery. The selective α_1 -adrenoceptor agonist (-)-phenylephrine, on the other hand, was equipotent with ($\pm$)-NA in the MCA and about two times less potent than ($\pm$)-NA in the MA. This agrees well with the range of potency differences suggested by Starke (1981) to be characteristic of α_1 -adrenoceptors. Although the potency of the selective α_1 -adrenoceptor agonist methoxamine was relatively low in the MCA, the difference in potency between the drug and (-)-NA was actually smaller than that reported for the postjunctional α_1 -adrenoceptors in the rabbit pulmonary artery (Starke et al. 1975).

Oxymetazoline has previously been reported to act selectively on α_2 -adrenoceptors (Starke et al. 1975, Docherty & McGrath 1980). In the rat MCA, oxymetazoline behaved as a partial agonist with a relatively high potency (three times that of ($\pm$)-NA). Unexpectedly, oxymetazoline also contracted the rat BA, which usually failed to respond to the other α -adrenoceptor agonists. This suggests that oxymetazoline has additional sites of action, unrelated to α -adrenoceptors, and therefore may be an unreliable tool in the classification of α -adrenoceptors in functional studies.

As pointed out by Starke (1981), the interpretation of results from agonist experiments is more difficult and less straightforward than is that from experiments with antagonists, due to differences in efficacy between agonists. In the present study, the selective α_1 -adrenoceptor antagonist prazosin was between three and four orders of magnitude more potent than the selective α_2 -adrenoceptor antagonist rauwolscine in inhibiting NA-induced contractions in the rat MCA and MA. Cohen et al. (1980) also found that prazosin was an extremely potent antagonist of NA-induced contractions in the rat MA. The present experimental results suggest that the antagonism between NA and prazosin was competitive in both the MCA and MA. This implies that the pA_2

values determined for prazosin in the two arteries may be considered equivalent to $-\log K_B$ (Arunlakshana & Schild 1959, Kenakin 1982). The pA_2 or K_B values estimated for prazosin in the MCA and MA largely agree with those reported by others for the interaction between prazosin and α_1 -adrenoceptors in both functional and binding studies (see Bylund & U'Prichard 1983, Steen et al. 1984). The weak effect of yohimbine on the contractile response to NA in the MCA further support the view that the predominant postjunctional α -adrenoceptor in this artery is of α_1 -type. In this respect the rat MCA is similar to cerebral arteries from man and monkey, which have also been reported to contain mainly α_1 -adrenoceptors at the post-junctional membrane (Toda 1983, Skärby & Andersson 1984). Notably, the slope of the Schild plot for rauwolscine in the rat MA was significantly lower than unity, and the pA_2 value for the antagonist (6.4) was substantially larger than that obtained in the MCA (5.4) and the pA_2 values reported in several other VSM preparations (5.3 - 5.9), containing mainly α_1 -adrenoceptors (see Steen et al. 1984). Therefore, the existence of a small population of postjunctional α_2 -adrenoceptors in the MA cannot be excluded.

In the rat BA, NA usually failed to elicit contraction even in the presence of propranolol and cocaine. This appears to exclude the possibility that the failure of the BA to respond to NA was caused by a predominance of β - over α -adrenoceptors in this artery or the presence of an efficient neuronal NA uptake mechanism. Rather, these findings seem to indicate a lack of contraction-mediating α -adrenoceptors in the BA. This interpretation is supported by binding studies demonstrating a paucity of α_1 -adrenoceptors in the rat BA, as compared with the rat tail artery (Silverberg et al. 1982). Notably the density of adrenergic nerves has similarly been reported to differ between the major cerebral arteries of the rat, the MCA being more richly supplied with adrenergic nerves than the BA (Kobayashi et al. 1981).

In agreement with the present study, Hirst et al. (1982) found that exogenously applied NA failed to elicit contraction in the rat BA. However, NA released by electrical field stimulation induced excitatory junctional potentials, sometimes associated

with the appearance of a spike potential and contraction (Hirst et al. 1982). Since the excitatory junctional potentials were resistant to inhibition by classical adrenoceptor blocking agents, it was postulated that the response was mediated by a new type of postjunctional adrenoceptors, termed γ -receptor, present only in the adrenergic neuroeffector region (Hirst et al. 1982). However, the γ -receptor has recently been questioned (Bevan 1984) and its existence remains to be established.

In conclusion, the results of the present study suggest that the contractile response to exogenous NA in the rat MCA and MA is mediated mainly by α_1 -adrenoceptors, although the involvement of a small population of postjunctional α_2 -adrenoceptors in the MA cannot be excluded. In contrast to the rat MCA, the BA appears to lack contraction-mediating α -adrenoceptors, indicating regional differences in the α -adrenoceptor distribution in the rat cerebrovascular bed.

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Table 1. Effects of cocaine and 6-hydroxydopamine (6-OHDA) pretreatment on the sensitivity of middle cerebral and mesenteric arteries to noradrenaline. Propranolol (3×10^{-7} M) was present in all experiments on the middle cerebral artery.

	Middle cerebral artery			Mesenteric artery		
	n	$-\log EC_{50}$	mean EC_{50} (M)	n	$-\log EC_{50}$	mean EC_{50} (M)
Control	7	5.61 ± 0.06	2.47×10^{-6}	7	5.41 ± 0.06	3.89×10^{-6}
Cocaine ^a	7	5.64 ± 0.05	2.28×10^{-6}	7	6.01 ± 0.03	9.82×10^{-7}
6-OHDA treated vessels	5	5.69 ± 0.10	2.03×10^{-6}	3	5.99 ± 0.08	1.02×10^{-6}

^a The middle cerebral artery was exposed to 10^{-6} M and the mesenteric artery to 10^{-5} M cocaine.

Table 2. Contractile effects of several α -adrenoceptor agonists in the middle cerebral artery. All $E_{\max}$ values are expressed as per cent of the maximum contractile response to noradrenaline. Propranolol (3×10^{-7} M) was present in all experiments.

Agonist	n	$E_{\max}$ (%)	$-\log EC_{50}$	Mean EC_{50} (M)	Potency ratio ^a
(⁺)-Noradrenaline	60	100	5.63 ± 0.02	2.32×10^{-6}	1.0
(-)-Noradrenaline	6	100 ± 2	5.89 ± 0.08	1.29×10^{-6}	1.8
(+)-Noradrenaline	7	> 42	< 4	$> 10^{-4}$	< 0.025
(-)-Adrenaline	8	110 ± 3	6.48 ± 0.10	3.33×10^{-7}	7.0
(-)-Phenylephrine	8	83 ± 5	5.51 ± 0.08	3.06×10^{-6}	0.76
Metoxamine	7	98 ± 8	4.85 ± 0.13	1.42×10^{-5}	0.16
Oxymetazoline	8	61 ± 8	6.12 ± 0.20	7.50×10^{-7}	3.1
Clonidine	5	> 10	< 4	$> 10^{-4}$	0.025

^a The potency ratio was obtained by dividing the mean EC_{50} value of the reference agonist (⁺)-noradrenaline by that determined for the other agonist.

Table 3. Results from the Schild plots for the antagonism of noradrenaline-induced contraction by prazosin and rauwolscine in middle cerebral and mesenteric arteries. The results are given as mean values with 95 % confidence interval within brackets. z represents the number of points used for calculating the regression line. Conditions as in figure 8 and 10.

Artery	Antagonist	z	Correlation coefficient	Slope of regression line	pA ₂ value
Middle cerebral artery	Prazosin	43	0.95	1.01 (0.90 - 1.12)	9.32 (9.17 - 9.49)
Middle cerebral artery	Rauwolscine	13	0.81	1.43 (0.74 - 2.11)	5.40 (5.03 - 5.63)
Mesenteric artery	Prazosin	40	0.97	0.93 (0.86 - 1.01)	9.70 (9.58 - 9.84)
Mesenteric artery	Rauwolscine	13	0.85	0.64 (0.38 - 0.89)	6.80 (6.47 - 7.39)

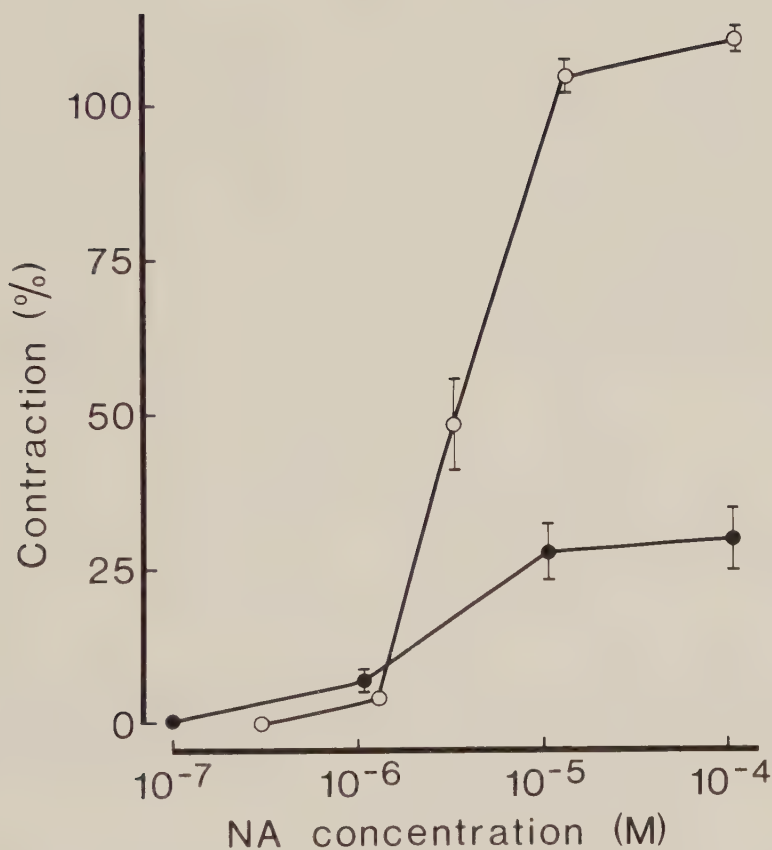


Fig 1. Concentration-response relationship for noradrenaline (NA) in middle cerebral (●, n=9) and mesenteric (○, n=6) arteries. Contractions are expressed as per cent of the contractile response to 124 mM K^+ .

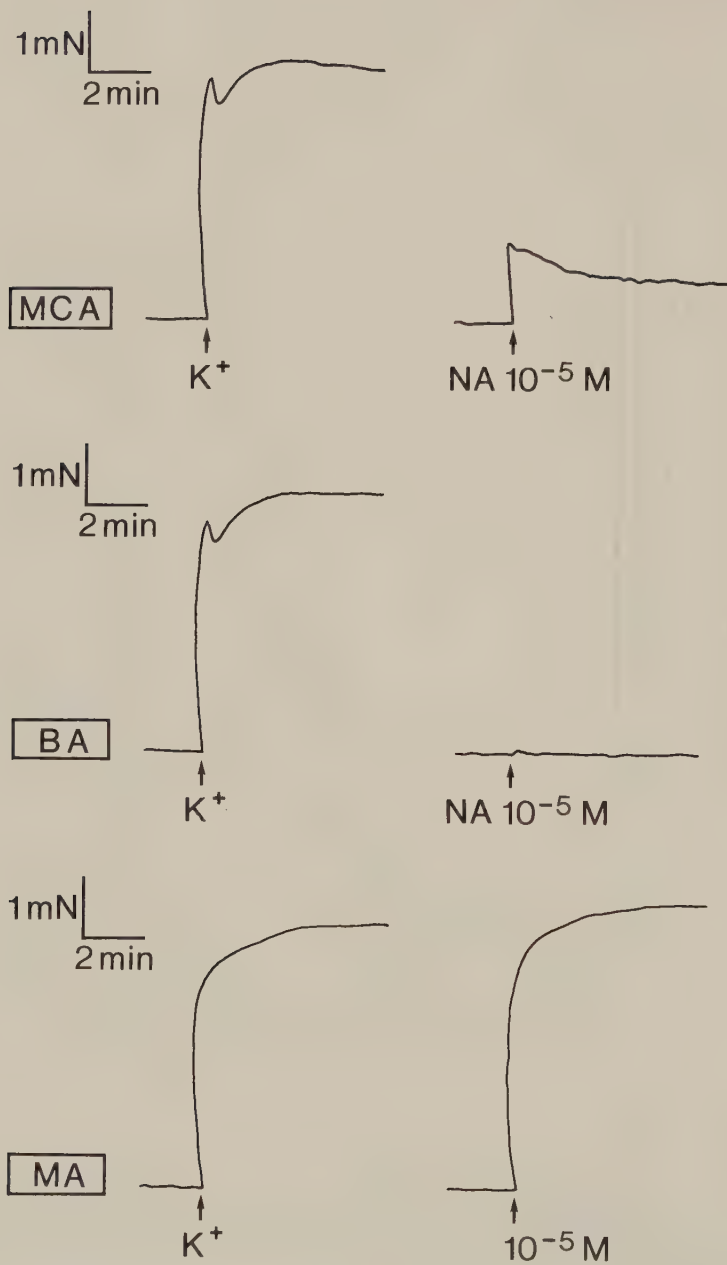


Fig 2. Contractile responses to 124 mM K^+ and $10^{-5}\ M$ noradrenaline (NA) in a middle cerebral (MCA), basilar (BA) and mesenteric (MA) artery.

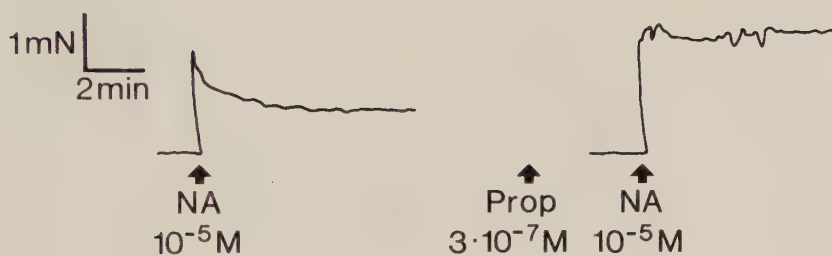


Fig 3. Contractile response to 10^{-5} M noradrenaline (NA) before and after application of 3×10^{-7} M propranolol (Prop) in a middle cerebral artery.

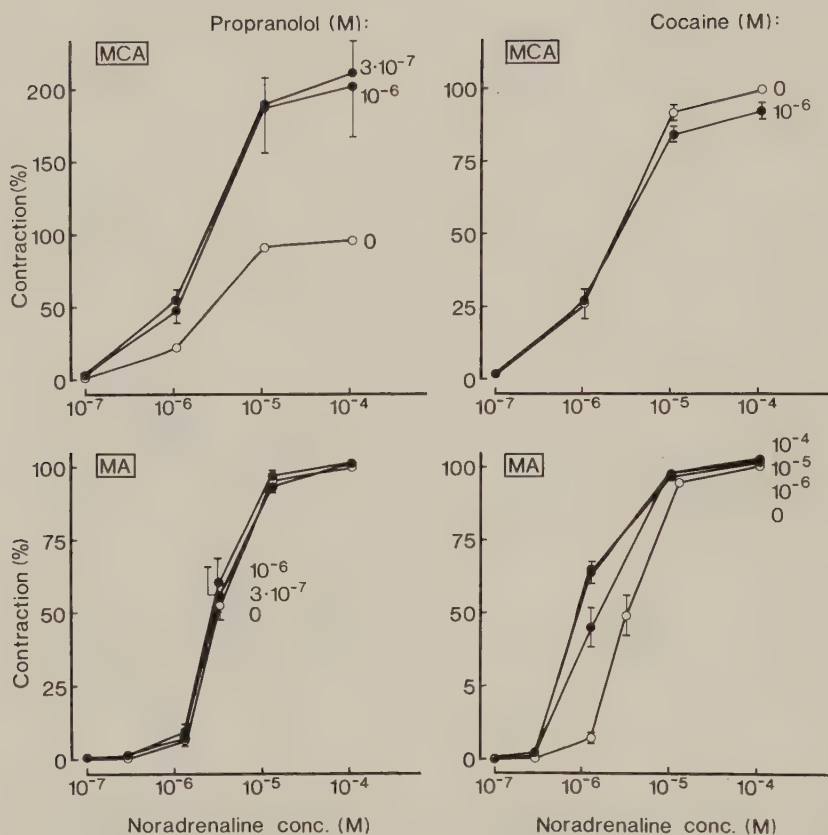


Fig 4. Effects of propranolol (left panel) and cocaine (right panel) on the concentration-response relationship for noradrenaline (NA) in middle cerebral (MCA, upper panel) and mesenteric (MA, lower panel) arteries. Open symbols refer to controls. When the effect of cocaine was examined in the MCA, propranolol (3×10^{-7} M) was included in the bath solution. The contractile response to 10^{-4} M NA in the control experiments was set to 100 %. Propranolol and cocaine were introduced into the bath solution approximately 20 min before application of NA. $n=3 - 10$.

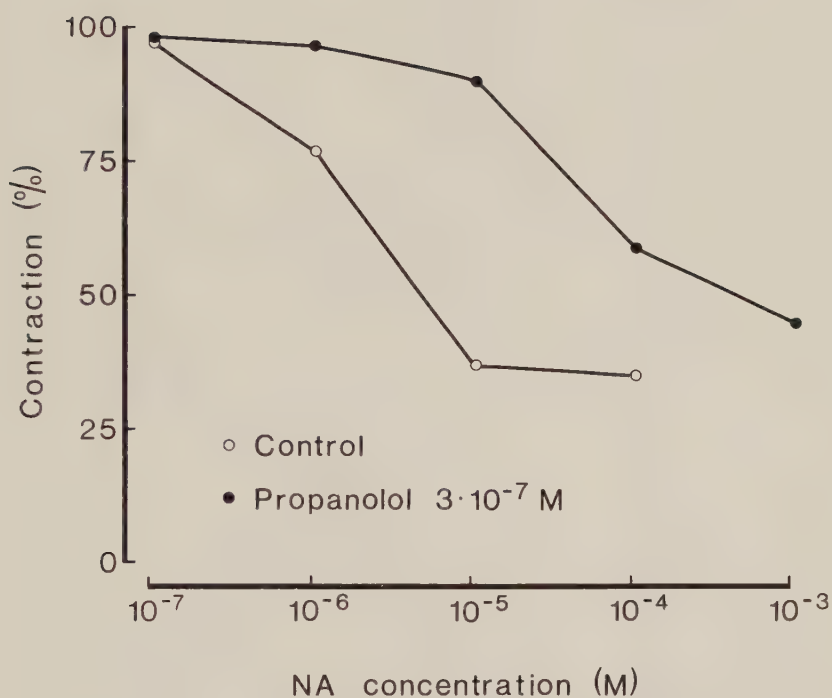


Fig 5. Relaxation induced by noradrenaline (NA) of a basilar artery contracted by 2.5×10^{-6} M prostaglandin $F_{2\alpha}$ in the absence (o) or presence (●) of 3×10^{-7} M propanolol. The amplitude of the prostaglandin $F_{2\alpha}$ -induced contraction immediately before application of NA was set to 100 %. NA was added cumulatively. The time of exposure to propanolol before addition of NA was approximately 15 min.

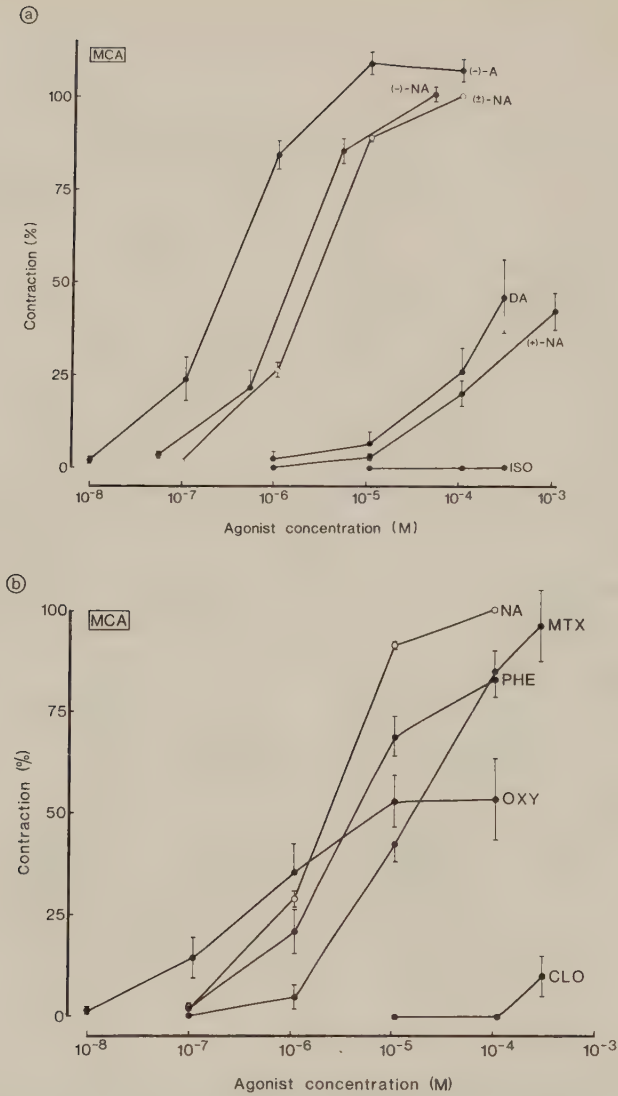


Fig 6 . (a) Contractile effects of ($\pm$)-noradrenaline (NA, $n=17$), (-)-NA ($n=6$), (+)-NA ($n=7$), (-)-adrenaline (A, $n=8$), dopamine (DA, $n=10$) and isoprenaline (ISO, $n=3$) in the middle cerebral artery (MCA). **(b)** Contractile responses to ($\pm$)-NA ($n=23$), oxymetazoline (OXY, $n=8$), (-)-phenylephrine (PHE, $n=8$), methoxamine (MTX, $n=7$) and clonidine (CLO, $n=5$) in the MCA. Contractions are expressed as per cent of the contractile response to 10^{-4} M ($\pm$)-NA. Propranolol (3×10^{-7} M) was present in all experiments.

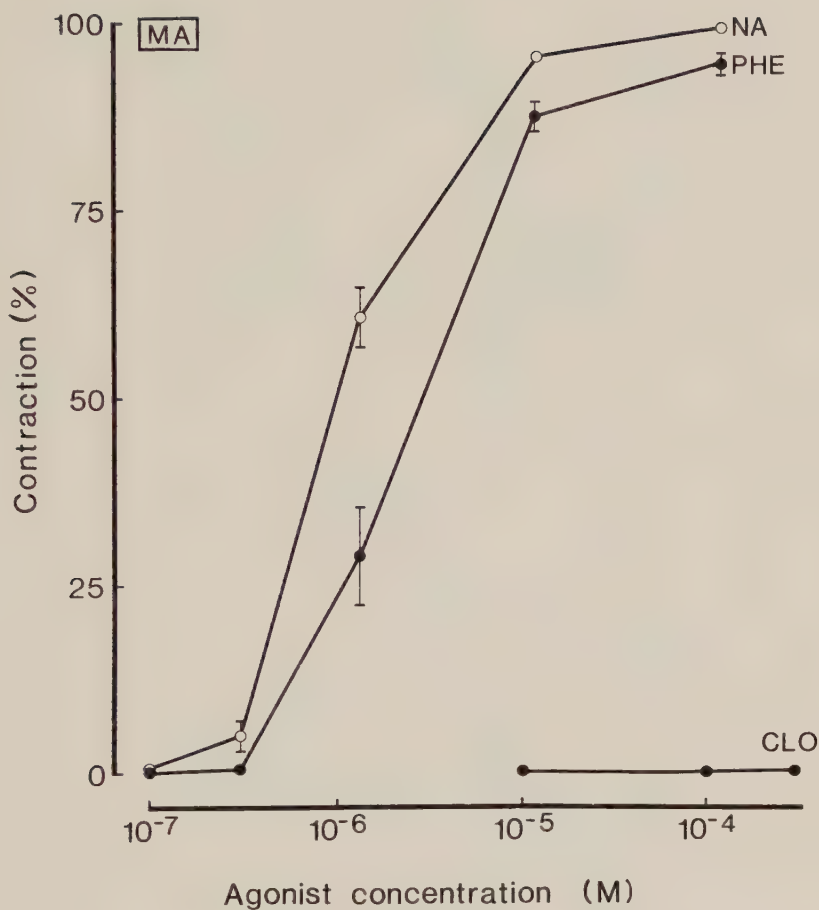


Fig 7. Contractile responses to ($\pm$)-noradrenaline (NA, $n=15$), (-)-phenylephrine (PHE, $n=9$) and clonidine (CLO, $n=5$) in the mesenteric artery (MA). Contractions are expressed as per cent of the contraction elicited by 10^{-4} M NA. Propranolol (3×10^{-7} M) and cocaine (10^{-5} M) were present throughout the experiments.

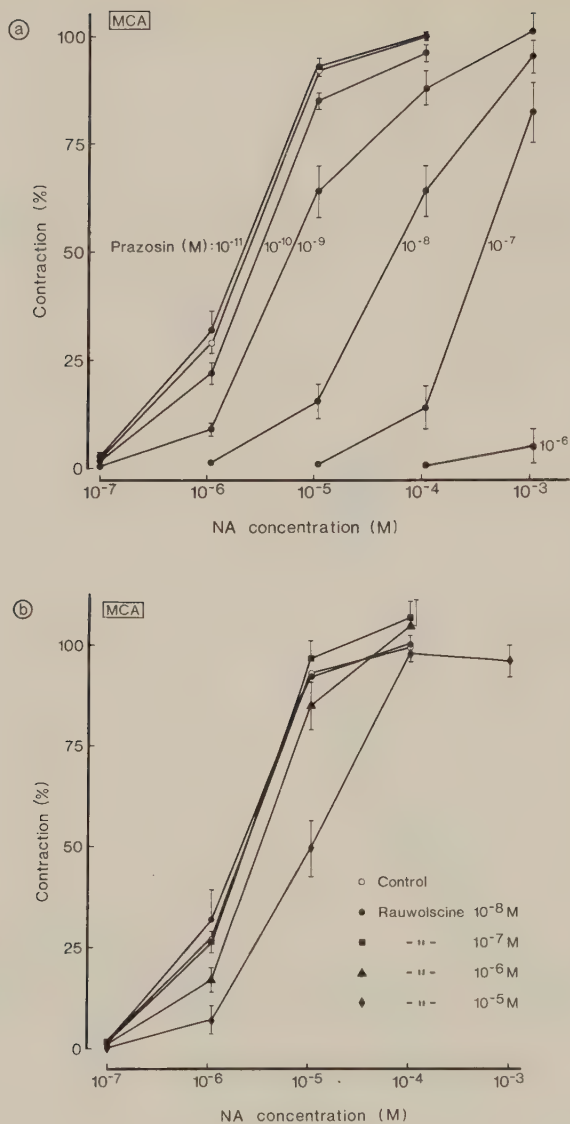


Fig 8 a, b. Effects of prazosin (a) and rauwolscine (b) on the concentration-response relationship for noradrenaline (NA) in the middle cerebral artery (MCA). The contractile response to 10^{-4} M NA in the absence of α -adrenoceptor antagonists was set to 100 % in all experiments. Open symbols refer to controls. Propranolol (3×10^{-7} M) was present throughout the experiments. The concentrations of prazosin (n=6 - 13) and rauwolscine (n=4 - 9) used are given in each panel.

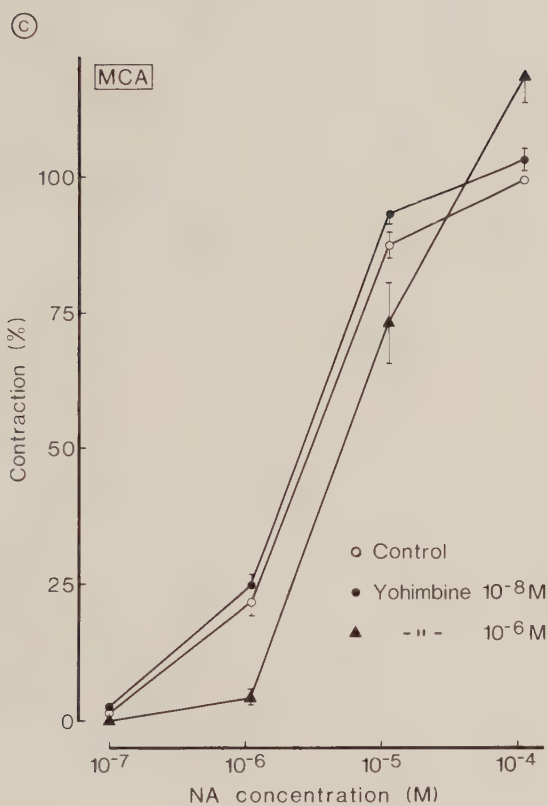


Fig 8c. Effect of yohimbine on the concentration-response relationship for noradrenaline (NA) in the middle cerebral artery (MCA). The concentrations of yohimbine used were 10^{-8} M (n=5) and 10^{-6} M (n=5). For further explanations, see legend to figure 8a,b.

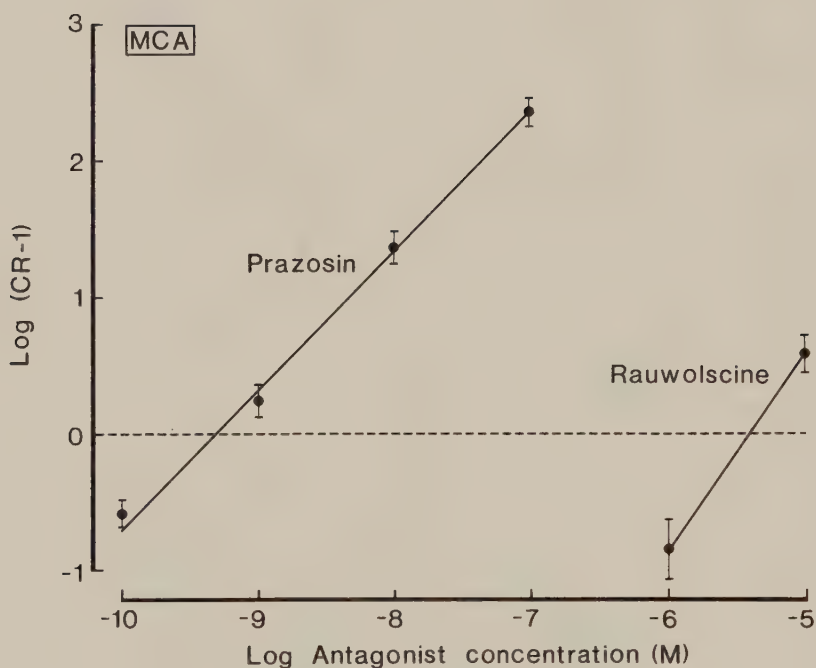


Fig 9. Schild plots for the antagonism of noradrenaline-induced contractions by prazosin and rauwolscine in the middle cerebral artery (MCA). The log (concentration ratio (CR) -1) is plotted against the log antagonist concentration (M) for the experiments shown in figure 8a, b. The characteristics of the regression lines are given in table 3.

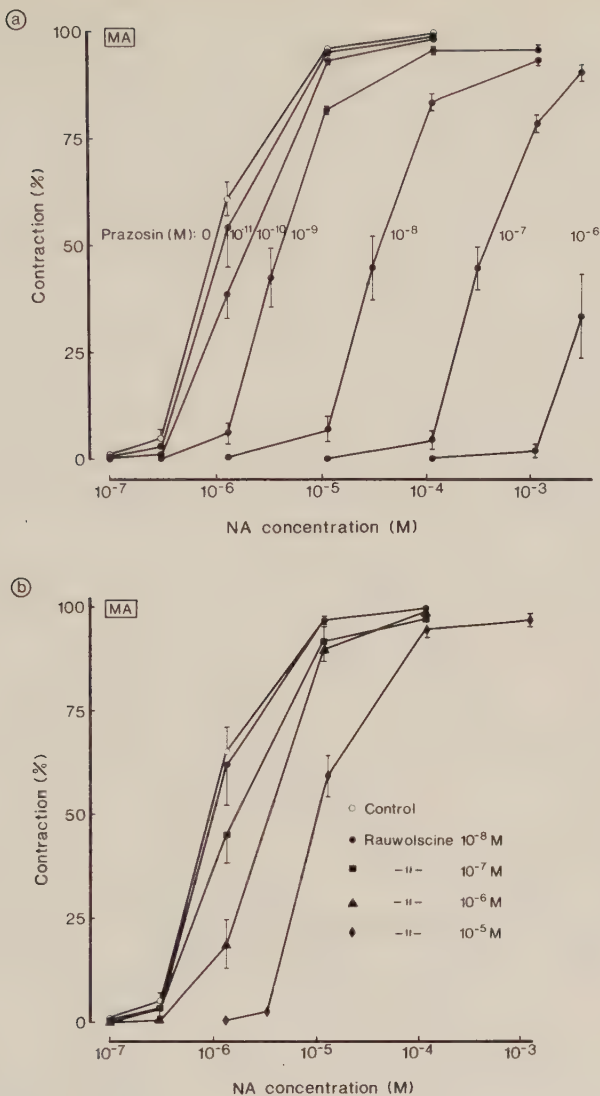


Fig 10. Effects of prazosin (a) and rauwolscine (b) on the concentration-response relationship for noradrenaline (NA) in the mesenteric artery (MA). The contractile response to 10^{-4} M NA in the absence of α -adrenoceptor antagonists was set to 100 % in all experiments. Open symbols refer to controls. Propranolol (3×10^{-7} M) and cocaine (10^{-5} M) were present throughout the experiments. The concentrations of prazosin ($n=4 - 12$) and rauwolscine ($n=3 - 5$) used are given in each panel.

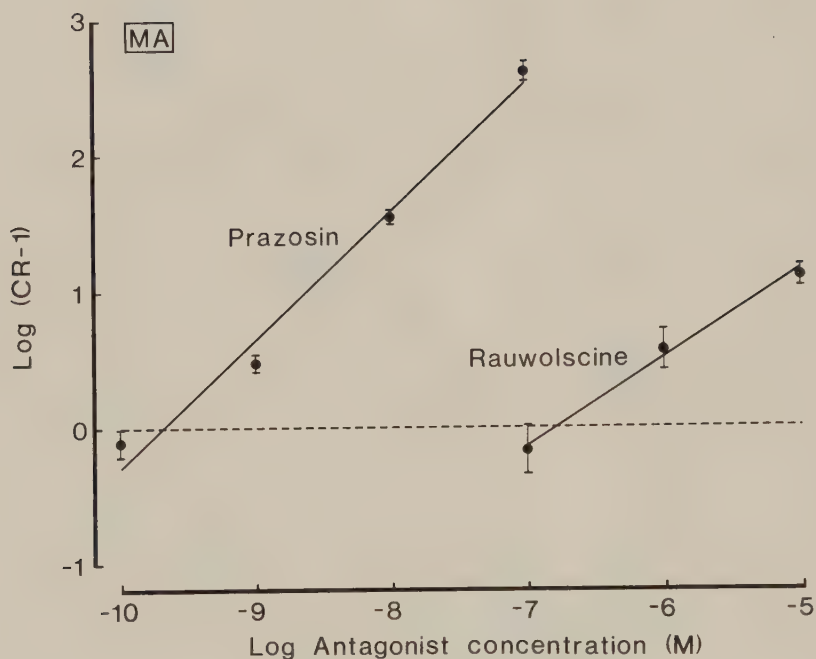


Fig 11. Schild plots for the antagonism of noradrenaline-induced contractions by prazosin and rauwolscine in the mesenteric artery (MA). The log (concentration ratio (CR) -1) is plotted against the log antagonist concentration (M) for the experiments shown in figure 10. The characteristics of the regression lines are given in table 3.

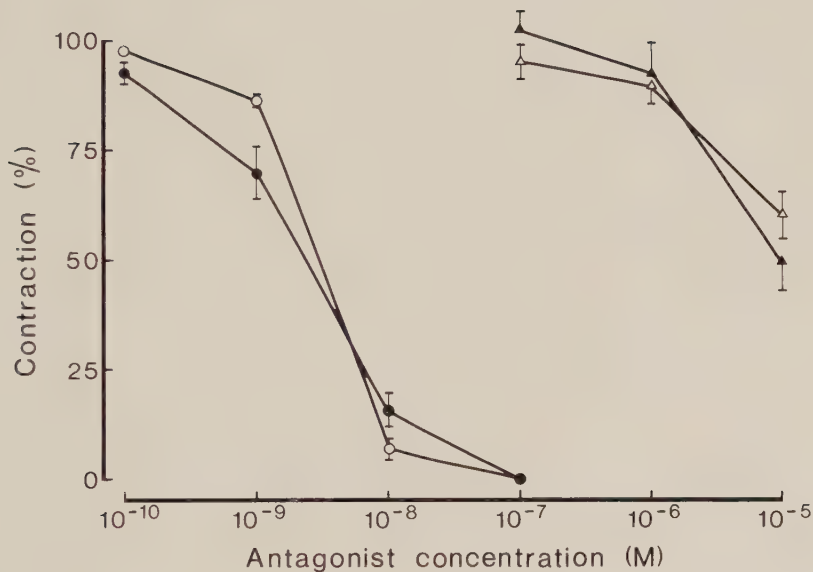


Fig 12. Inhibition of the contractile response to 10^{-5} M noradrenaline (NA) by prazosin (circles) and rauwolscine (triangles) in middle cerebral (filled symbols) and mesenteric (open symbols) arteries. The degree of inhibition was calculated from the concentration-response curves for NA before and after application of the α -adrenoceptor blockers for the same experiments as those shown in figure 8a, b and 10. The amplitude of the contraction induced by 10^{-5} M NA in the absence of α -adrenoceptor antagonists was set to 100 %.

VI

**INFLUENCE OF EXTRACELLULAR CALCIUM AND NIFEDIPINE
ON α_1 - AND α_2 -ADRENOCEPTOR-MEDIATED CONTRACTILE RESPONSES
IN ISOLATED RAT AND CAT CEREBRAL AND MESENTERIC ARTERIES**

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Running title: Vascular α -adrenoceptor subtypes and calcium

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SUMMARY

T. Skärby, E. D. Högestätt and K.-E. Andersson: Influence of extracellular calcium and nifedipine on α_1 - and α_2 -adrenoceptor mediated contractile responses in isolated rat and cat cerebral and mesenteric arteries.

The influence of extracellular Ca^{2+} and nifedipine on contractile responses to $10\text{ }\mu\text{M}$ noradrenaline (NA) was investigated in isolated rat and cat middle cerebral (RMCA, CMCA) and mesenteric (RMA, CMA) arteries. In the CMCA (containing predominantly α_2 -adrenoceptors), the NA-induced contractions developed considerably slower than in the RMCA, RMA (containing mainly α_1 -adrenoceptors) and CMA (sensitive to both α_1 - and α_2 -adrenoceptor selective antagonists). The tonic component of the NA-induced contraction in the four types of artery was substantially suppressed after only short periods in Ca^{2+} -free solution. The contractile response to 124 mM K^+ in each type of artery, excluding the CMCA, was more sensitive to Ca^{2+} deprivation than was that to NA. This suggests that NA, besides mobilizing extracellular Ca^{2+} , also can release Ca^{2+} from an intracellular pool in the RMCA, RMA and CMA, but not in the CMCA. Thus, α_1 -adrenoceptor-mediated contractions in the RMCA and RMA seem to depend on both Ca^{2+} influx and intracellular Ca^{2+} release, whereas α_2 -adrenoceptor-mediated contractile responses in the CMCA appear to rely almost entirely on Ca^{2+} influx. Both the maximum response and the tonic component of the NA-induced contraction were significantly more sensitive to nifedipine in the CMCA than in the RMCA. In comparison with the NA-induced contractions in these arteries, those in the RMA and CMA were relatively resistant to nifedipine. Notably, the maximum inhibition of the tonic component produced by nifedipine appeared to be inversely related to the amplitude of the contraction elicited by NA in Ca^{2+} -free medium. The nifedipine resistant part of the contractile response to NA in the different arteries was not appreciably influenced (RMA, RMCA) or only slightly reduced (CMA, CMCA) by prior K^+ depolarization. In the CMCA exposed to NA in Ca^{2+} -free medium, nifedipine almost abolished the contraction induced by readdition of Ca^{2+} , whereas in the other types of artery, Ca^{2+} reapplication evoked a significant contraction also in the presence of the

drug. The differential effects of nifedipine presumably reflect differences between the arteries not only in the relative contribution of Ca^{2+} influx and intracellular Ca^{2+} release to the contractile activation, but also in the nifedipine sensitivity of the Ca^{2+} entry pathways utilized by NA. It is concluded that the mechanism through which NA induces contraction seems to be related both to the subtype of α -adrenoceptor stimulated by NA and to the anatomical origin of the vessel.

Key words: Vascular smooth muscle; excitation-contraction coupling; α -adrenoceptors; extracellular calcium; nifedipine

INTRODUCTION

Both α_1 - and α_2 -adrenoceptors have been demonstrated post-junctionally in vascular smooth muscle (VSM); stimulation of either type of receptor leading to contraction (Drew & Whiting, 1979; Timmermans et al., 1979; Docherty and Mc Grath, 1980; Skärby et al., 1981; Högestätt & Andersson, 1984). Much interest has recently been focused on the mechanisms providing activator Ca^{2+} to the contractile proteins after α_1 - and α_2 -adrenoceptor stimulation. In pithed rats and cats, ganglion-blocked rabbits (Van Zwieten et al., 1982) and in the isolated dog hindlimb (Llenas & Massingham, 1983), vasopressor responses induced by selective α_1 -adrenoceptor agonists, but not those elicited by selective α_2 -adrenoceptor agonists, were shown to be relatively resistant to organic " Ca^{2+} entry blockers" (Fleckenstein 1977). These observations led to the suggestion that α_1 -adrenoceptors in VSM are coupled to release of intracellularly stored Ca^{2+} , whereas stimulation of α_2 -adrenoceptors promotes the influx of Ca^{2+} from the extracellular medium (see Van Meel, 1982). The experiments on which this conclusion was based were largely conducted on whole animal preparations. However, studies of isolated blood vessels have resulted in divergent conclusions (DeMey & Vanhoutte, 1981; Cauvin et al., 1982, Caverio et al., 1983, Godfraind et al., 1982).

In order to further elucidate the cellular mechanisms coupled to postjunctional α_1 - and α_2 -adrenoceptors and to appreciate regional differences in these mechanisms, we compared the effects of Ca^{2+} deprivation and nifedipine on contractile responses induced by noradrenaline (NA) in isolated rat middle cerebral and mesenteric arteries (α_1) and cat middle cerebral (α_2) and mesenteric (sensitive to both α_1 - and α_2 -adrenoceptor selective antagonists) arteries.

Vascular preparations

Rats (Sprague-Dawley) and barbiturate anaesthetized cats (pentobarbital, 30 mg/kg i.p.) of either sex were decapitated and the brain and part of the mesenterium rapidly removed and put into cold (8 - 12°C) "standard" Krebs solution (for composition, see below). Rat and cat middle cerebral (RMCA, CMCA) and mesenteric (RMA, CMA) arteries were subsequently dissected out under a microscope. The outer diameter of the excised arteries decreased in the following order: CMA (≈ 0.5 mm; third generation of branches originating from the superior CMA) > CMCA (≈ 0.4 mm) > RMA (≈ 0.2 mm; second generation of branches originating from the superior RMA) > RMCA (≈ 0.1 mm). The vessels were either used immediately or stored in a refrigerator (8 - 12°C) for use within 24 hours.

Experimental procedure

Ring segments of the arteries (1 - 3 mm long) were suspended between two metal holders (diameter = 50 - 200 μ M) in small volume (2.5 - 5 ml) vessel baths. The mechanical activity was recorded "isometrically" by means of Grass Instruments FT03C force-displacement transducers, and the output from the transducers was displayed on a Grass Instruments polygraph. The temperature of the buffer solution in the vessel baths was kept at 37°C. The bath fluid was continuously bubbled with a mixture of O₂ (95 %) and CO₂ (5 %), resulting in a pH of approximately 7.4. During an equilibration period of one hour, the vessels were frequently stretched until a stable resting tension of approximately 2 mN (rat arteries) or 4 mN (cat arteries) was obtained. For further experimental details see Högestätt et al. (1983) and Skärby et al. (1983). The preparations were not accepted for further experiments unless at least two reproducible contractions induced by NA (10 μ M) or an isotonic K⁺-Krebs solution (for composition, see below) were first obtained. Responses were considered reproducible when the variation between consecutive contractions was 10 % or less. Propranolol (0.3 μ M) was included in the bath solution when the response to NA was examined in order to block β -adrenoceptor-mediated effects of the agonist. The contraction induced by NA in standard Krebs solution will hereafter be

referred to as the control response.

Buffer solutions

1. The standard Krebs solution contained (in mM): NaCl 119, NaHCO₃ 15, KCl 4.6, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.5, glucose 11.
2. The isotonic K⁺-Krebs solution (124 mM K⁺) was prepared by substituting all NaCl in the standard Krebs solution with equimolar amounts of KCl.
3. Ca²⁺-free solutions were prepared by substituting all CaCl₂ with 10 μM EGTA.

Drugs

(±)-Noradrenaline HCl (Sigma), rauwolscine HCl (Roth) and cocaine HCl (Merck) were dissolved in 0.9 % NaCl, containing 0.1 mM ascorbic acid. Prazosin (Pfizer) was dissolved in 10 % lactic acid, giving a final prazosin concentration of 1 mM. Nifedipine (0.1 mg/ml) (Bayer) and propranolol HCl (1 mg/ml) (ICI) were provided in ampoules. Nifedipine was kept dark until used to avoid light-induced degradation. All drugs except those supplied in ampoules were prepared as stock solutions and stored at -70° C until used. Fresh dilutions of the drugs were always prepared on the day of experiment in 0.9 % NaCl, containing 0.1 mM ascorbic acid. All concentrations given in "Results" refer to the final bath concentrations.

Statistics

Student's t-test (two-tailed) was used to determine statistical significance and the 0.05 level of probability was accepted as significant. However, the effects of nifedipine were compared by analysis of variance, and in subsequent t-tests, statistical significance was accepted only when $P < 0.01$. The least squares method was used for calculating linear regressions. The results shown in tables, figures and the text are expressed as mean values $\pm$ SEM, with n referring to the number of vessels examined.

RESULTS

Effect of prazosin and rauwolscine on the contractile response to NA

The amplitude of the contractions induced by 10 μM NA in the RMCA, RMA, CMCA and CMA corresponded to between 90 % and 95 % of that elicited by 0.1 mM NA. When expressed as a percentage of contractile response to 124 mM K^+ , the contraction induced by 10 μM NA was $97 \pm 4 \%$ ($n=9$) in the RMA, $109 \pm 2 \%$ ($n=25$) in the CMA, $48 \pm 3 \%$ ($n=13$) in the RMCA and $44 \pm 2 \%$ ($n=20$) in the CMCA.

As can be seen in Figs. 1 and 2, NA (10 μM) induced an initial rapid tension increase followed by a tonic response in the RMCA, RMA and CMA. However, in the CMCA, the NA-induced contractions developed considerably slower than in the other types of artery. The contractions usually stabilized after 5 min, and the amplitude of the response at this time will hereafter be referred to as the tonic component. Figs. 1 and 2 also show the effects of prazosin (1 nM - 10 μM) and rauwolscine (1 nM - 10 μM) on the contractile responses to NA in the four types of artery. Although the potencies of the α -adrenoceptor blockers differed markedly between the arteries, prazosin and rauwolscine influenced the time course of the NA-induced contractions similarly in each type of vessel.

Contractile responses to NA and K^+ in Ca^{2+} -free medium

Omission of Ca^{2+} from the bath solution consistently suppressed the contractile response to NA (10 μM) in all four types of artery. The reduction of the maximum response and the tonic component of the NA-induced contraction after various time periods in Ca^{2+} -free Krebs solution is shown in Fig. 3. As can be seen, the tonic component was substantially reduced by Ca^{2+} deprivation in all four vessel types. The amplitude of the maximum response to 10 μM NA after 10 min in Ca^{2+} -free medium decreased in the following order between the various vessel segments: CMA $\approx$ RMA > RMCA >> CMCA; the NA-induced contraction in the CMCA was virtually abolished after this treatment. Excluding the CMCA, in which the responses to K^+ and NA were equally reduced by a 10-min preincubation in Ca^{2+} -free medium, the K^+ -induced contraction was significantly more depressed by Ca^{2+} deprivation than was the

response to NA (Fig 4).

Effects of nifedipine on K^+ - and NA-induced contractions

Nifedipine effectively relaxed K^+ - induced contractions in the RMCA and CMCA (Fig. 5). Neither the RC_{50} nor the R_{max} values differed significantly between the two arteries (Table 1). The contractile response to NA (10 μ M), on the other hand, was more sensitive to nifedipine in the CMCA than in the RMCA. This was true both for the maximum response and for the tonic component of the NA-induced contraction (Fig. 6, Table 2). In contrast to the NA-induced contractions in the RMCA and CMCA, those in the RMA and CMA were relatively resistant to nifedipine (Fig. 6, Table 2). Furthermore, in the RMCA and RMA, but not in the CMCA and CMA, the tonic component of the contractile response to NA was more inhibited by nifedipine than was the maximum response (Fig. 6a, Table 2). This difference was particularly apparent in the RMCA, which in the presence of 0,3 μ M nifedipine responded to NA with a rapid phasic contraction followed by a tonic response of lower amplitude. Interestingly, nifedipine was approximately equipotent in inhibiting NA-induced contractions and in relaxing contractions evoked by K^+ in the CMCA. However, in the RMCA, the contractile response to K^+ was slightly more sensitive to nifedipine than either the maximum response or the tonic component of the NA-induced contraction.

On the basis of the maximum contractile response elicited by 10 μ M NA, the arteries could be ranked in the following order with respect to their ability to contract in the presence of nifedipine: RMA $\approx$ CMA $\gg$ RMCA $\gg$ CMCA. As demonstrated by regression analysis, a close correlation ($r=0.99$; $P < 0.01$) was found between the degree to which 3 μ M nifedipine inhibited the tonic component and the maximum amplitude of the NA-induced contraction after 10 min in Ca^{2+} -free medium (Fig 7). The inhibition produced by nifedipine in the different arteries was reversible, as frequent washes in drug-free solution restored the NA response.

Contractile response to NA in K^+ -depolarized muscle

The isotonic K^+ - Krebs solution evoked strong and sustained contractions in all four types of artery. However,

preparations preincubated with 0.3 μM nifedipine responded to K^+ with a transient contraction, which stabilized after 5 to 10 min at a level slightly above the baseline tension. As shown in Fig. 8, the nifedipine resistant part of the NA-induced contractions was unaltered (RMCA and RMA) or only slightly reduced (CMCA and CMA) by prior K^+ -depolarization; the maximum response to NA in K^+ -depolarized preparations was $98 \pm 5\%$ ($n=6$) in the RMCA, $103 \pm 4\%$ ($n=8$) in the RMA, $69 \pm 2\%$ ($n=6$) in the CMCA and $87 \pm 3\%$ ($n=6$) in the CMA, as compared with that elicited by NA before application of K^+ (also obtained in the presence of nifedipine).

Effect of nifedipine on Ca^{2+} -induced contractions in NA-exposed arteries

In a separate series of experiments, all four types of artery were repeatedly exposed to NA (10 μM) in Ca^{2+} -free Krebs solution until the NA response was reduced to a minimum; a small sustained contraction, less than 5 % of the control response, sometimes persisted. Reapplication of Ca^{2+} (1.5 mM) to the RMA and CMA, exposed to NA, induced a rapid and sustained contraction (Fig. 9). The amplitudes of the Ca^{2+} -evoked contractions in these arteries amounted to $98 \pm 1\%$ ($n=6$) and $90 \pm 2\%$ ($n=6$) of the control response. If nifedipine (0.3 μM) was introduced into the bath solution 15 to 20 min before Ca^{2+} , it suppressed the Ca^{2+} -induced contractions by $33 \pm 4\%$ ($n=6$) and $67 \pm 1\%$ ($n=6$) in the RMA and CMA, respectively. Nifedipine also delayed the onset of the contractile response to Ca^{2+} by 15 to 45 seconds and made the tension increase less rapidly.

The contractile responses to 1.5 mM Ca^{2+} in the " Ca^{2+} -deprived" RMCA and CMCA exposed to NA were more complex and less reproducible than those obtained in the RMA and CMA. As shown in Fig. 9, the Ca^{2+} -induced contractions were composed of an initial phasic response followed by a tonic component. The peak tension of the initial transient contraction, relative to the control response, was $65 \pm 15\%$ ($n=6$) in the RMCA and $46 \pm 12\%$ ($n=6$) in the CMCA. In the RMCA, the tonic component of the Ca^{2+} -induced contraction usually required 10 to 20 min to be fully developed (not shown). A similar slow tension increase was not observed in the CMCA. Nifedipine (0.3 μM) almost abolished the Ca^{2+} -induced

contraction in the CMCA (n=4). However, in the RMCA, a significant portion of the response still remained in the presence of the drug. The amplitude of this residual contraction amounted to $41 \pm 4 \%$ (n=6) of the control response (elicited by NA in standard Krebs solution).

DISCUSSION

The postjunctional α -adrenoceptors in the RMCA, RMA, CMCA and CMA have recently been characterized by means of subtype selective α -adrenoceptor agonists and antagonists. Thus, it was suggested that the postjunctional α -adrenoceptors in the RMCA and RMA are mainly of the α_1 -type (Högestätt & Andersson, 1984). Using radio-ligand binding technique, Colucci et al. (1980, 1981) came to a similar conclusion regarding the α -adrenoceptor population in rat mesenteric arteries. Skärby et al. (1981, 1983) provided evidence suggesting that the α -adrenoceptors in the CMCA are predominantly of the α_2 -type, and this view was supported by the findings of Medgett and Langer (1983). The CMA, on the other hand, was suggested to contain a "broader" type of α -adrenoceptor, unable to differentiate between antagonists that in other tissues act preferentially on α_1 - or α_2 -adrenoceptors (Skärby et al. 1983). Since each of the arteries examined in the present study was supplied with a relatively homogenous population of α -adrenoceptors, we could use the non-selective agonist NA to rather selectively stimulate either α_1 - or α_2 -adrenoceptors on the VSM cells.

Components of the NA-induced contraction

The contractile response to NA in the RMCA, RMA and CMA could usually be divided into an initial fast and an ensuing tonic component (cf. Bohr, 1963, Watkins & Davidson, 1980). However, in the CMCA, NA produced mainly a monophasic slow tension increase. In the pithed rat preparation, Timmermans & van Zwieten (1980) and van Meel et al. (1981) described that the pressor response elicited by selective α_2 -adrenoceptor agonists developed more slowly than that induced by selective α_1 -adrenoceptor agonists. From these observations, it was speculated that the fast component and the slow component of the contractile response induced by nonselective α -adrenoceptor agonists in the isolated rabbit aorta were caused by stimulation of α_1 - and α_2 -adrenoceptors, respectively (van Meel et al., 1981). This implies that the two contraction components should be affected differently by selective α -adrenoceptor blockers. As shown in the present study, prazosin and rauwolscine affected the time course of the NA-induced contraction similarly in each type of artery. Furthermore, even

though the postjunctional α -adrenoceptors in the rabbit aorta, RMCA and RMA have been classified as being predominantly of the α_1 -type (Docherty et al., 1981, Högestätt & Andersson, 1984), the contractions induced by NA could still be resolved into two components in these vessels. These findings do not favour the view that the two components of the contractile response to NA are attributable to stimulation of different α -adrenoceptor subtypes. Nevertheless, the rate of tension development induced by NA was considerably slower in the CMCA (containing predominantly α_2 -adrenoceptors) than in the RMCA and RMA (mainly endowed with α_1 -adrenoceptors), suggesting differences in the excitation-contraction coupling process associated with stimulation of the two receptor subtypes.

Ca²⁺ pools used for activation

The fast and slow components of NA-induced contractions seen in several VSM preparations have been attributed to mobilization of different Ca²⁺ pools (Steinsland et al., 1973; Deth & van Breemen, 1974, Bolton 1979). As shown in the different arteries presently studied, the main part of the tonic component of the contractile response to NA was abolished after only short incubation periods in Ca²⁺-free solution. This suggests that the Ca²⁺ utilized for contraction during the tonic component is derived mainly from the extracellular medium, irrespective of the α -adrenoceptor subtype stimulated by NA.

High K⁺ solutions are generally believed to induce VSM contraction by enhancing Ca²⁺ entry from the extracellular medium (Bolton, 1979). The observation that the contractile response to K⁺ in the RMCA, RMA and CMA was reduced to a larger extent by Ca²⁺ deprivation than was that elicited by NA argues that NA, in addition to mobilizing extracellular Ca²⁺, also can liberate Ca²⁺ from a more tightly bound pool (hereafter referred to as intracellular Ca²⁺) in these arteries. These results do not support the view that α_1 -adrenoceptors are linked specifically to either of these processes. A similar conclusion was reached by Cauvin et al. regarding the α_1 -adrenoceptors in the rabbit aorta (1982). However, in the CMCA, K⁺- and NA-induced contractions were equally suppressed and almost abolished after the same treatment

(present study), suggesting that the contractile response to NA in this preparation rely almost entirely on influx of Ca^{2+} from the extracellular medium. The failure of NA to liberate intracellularly stored Ca^{2+} in the CMCA might reflect a general lack of coupling between α_2 -adrenoceptors and intracellular Ca^{2+} release, as suggested by, e.g., Van Meel (1982).

As judged from the amplitude of the contractile response to NA in Ca^{2+} -free medium, the amount of intracellular Ca^{2+} released by NA appeared to be smaller in the RMCA than in the CMA and RMA, and almost negligible in the CMCA. In addition, the degree of intracellular Ca^{2+} release does not seem to be related to the calibre of the vessel, but rather to the region from which the artery was excised, as NA-induced contractions in mesenteric arteries were always more resistant to Ca^{2+} deprivation than were those in cerebral arteries. Evidence of regional differences in the excitation-contraction coupling process between cerebral and peripheral arteries has previously been reported for both animal and man (Allen & Banghart, 1979; Shimizu et al., 1980; Brandt et al., 1981; McCalden and Bevan, 1981; Towart, 1981; Bevan, 1983; Yamamoto et al., 1983).

Differential effects of nifedipine on NA- and K^{+} -induced contractions

Analogous with its mechanism of action on heart muscle, the relaxing effect of nifedipine on VSM has been explained by inhibition of Ca^{2+} entry (Fleckenstein, 1977; Andersson & Högestätt, 1984). Although Ca^{2+} entry blockers, such as nifedipine, verapamil and diltiazem, are commonly considered to be relatively selective inhibitors of Ca^{2+} influx through potential-operated Ca^{2+} channels (POCs) (Bolton, 1979; Andersson & Högestätt 1984), recent studies suggest that receptor-operated Ca^{2+} channels (ROCs) in certain VSM preparations may also be effectively blocked by these drugs (Walus et al., 1981; Cauvin et al., 1982; Bevan 1983).

In the present study, contractions elicited by NA in the RMA and CMA were relatively resistant to nifedipine. Although the tonic component of the NA-induced contraction in these vessels was substantially suppressed by Ca^{2+} deprivation, $0.3 \mu\text{M}$

nifedipine (a concentration producing a maximum relaxation of K^+ -induced contractions in the RMCA and CMCA) only reduced the tonic response to NA by approximately 30 %. Moreover, the nifedipine-resistant part of the NA-induced contraction appeared to be relatively independent of the state of polarization of the cell membrane, as it was unaffected (RMA) or only slightly reduced (CMA) by prior K^+ -depolarization. Reintroduction of Ca^{2+} to the RMA and CMA, which had been depleted of Ca^{2+} and exposed to NA, induced sustained and reproducible contractions of approximately the same magnitude as those elicited by NA in standard Krebs solution. Although the Ca^{2+} -induced contractions may be regarded as reflecting Ca^{2+} influx only, nifedipine ($0.3 \mu M$) still failed to abolish the contractile response to Ca^{2+} in the RMA and CMA. These results are compatible with the view that NA can activate a population of ROCs resistant to nifedipine in these arteries.

Nifedipine effectively relaxed the K^+ -contracted RMCA and CMCA, and the relaxation produced did not differ significantly between the two arteries. However, the NA-induced contraction (both the maximum response and the tonic component) was more sensitive to nifedipine in the CMCA than in the RMCA. Pronounced regional differences were also observed, as the maximum response and tonic component of the NA-induced contraction in the RMCA and CMCA were considerably more sensitive to nifedipine than were those in the corresponding mesenteric arteries with respect to both the I_{max} and IC_{50} values. Considering that the size of the RMA and RMCA was smaller than that of the CMCA, these findings do not agree with the observations by Godfraind and co-workers, who showed that the degree of reduction of the contractile response to NA by a putative Ca^{2+} entry blocker was inversely related to the calibre of the vessel (Broekaert & Godfraind 1979, Godfraind & Dieu 1981).

Interestingly, NA- and K^+ -induced contractions in the CMCA were similarly sensitive to both nifedipine and Ca^{2+} removal. In addition, nifedipine almost abolished the contraction induced by Ca^{2+} readdition in arteries exposed to NA in Ca^{2+} -free medium. These findings may indicate that K^+ and NA largely utilize the same pathway to increase the myoplasmic Ca^{2+}

concentration. Electrophysiological studies on isolated cat cerebral arteries have shown a close correlation between NA-induced contraction and membrane depolarization (Harder et al., 1981; Harder, 1983). It therefore seems possible that nifedipine inhibited the contractile response to NA in the CMCA by blocking POCs, activated by NA-induced membrane depolarization.

In the RMCA, the maximum response of the NA-induced contraction was less inhibited by nifedipine than the tonic component. Release of intracellularly stored Ca^{2+} during the initial fast component of the NA-induced contraction, can probably explain this difference. The tonic component of the contractile response to NA was only slightly less sensitive to nifedipine than was the K^{+} -induced contraction. Although electrophysiological evidence is lacking, it may be speculated that part of the tonic component of the NA-induced contraction in the RMCA was caused by membrane depolarization and subsequent influx of Ca^{2+} through POCs. However, three pieces of evidence indicate the existence of a nifedipine resistant Ca^{2+} entry pathway, possibly equivalent to a ROC, in the RMCA. First, nifedipine failed to abolish the tonic component of the contractile response to NA. Secondly, the nifedipine resistant part of the NA-induced contraction was present also after prior K^{+} depolarization. Thirdly, the contraction induced by reintroduction of Ca^{2+} to the Ca^{2+} -depleted RMCA was not abolished by nifedipine.

The sensitivity of the NA-induced contraction (both the maximum response and tonic component) to nifedipine decreased in the following order between the different arteries: CMCA >> RMCA >> CMA $\approx$ RMA. This differential effect of nifedipine may reflect differences between the vessels not only in the extent of intracellular Ca^{2+} release, but also in the nifedipine sensitivity of the Ca^{2+} entry pathways utilized by NA. Interestingly, the maximum inhibition of the tonic component produced by nifedipine appeared to be correlated to the amplitude of the contraction elicited by NA in Ca^{2+} -free medium. This suggests that the nifedipine resistant part of the tonic component somehow is related to the extent to which NA releases intracellular Ca^{2+} in that vessel, i.e. the greater the intracellular Ca^{2+} release, the greater the nifedipine resistance

of the tonic response. Such a correlation would be anticipated if the nifedipine resistant part of the tonic component reflects a fast refilling of the NA sensitive intracellular Ca^{2+} store directly from the extracellular medium and subsequent release of Ca^{2+} into the myoplasm. Casteels and Droogmans (1981) have previously suggested that such a pathway, resistant to Ca^{2+} entry blockers, may represent an alternative model of the ROC.

In conclusion, the results of the present study suggest that stimulation of vascular α_1 -adrenoceptors in the RMCA and RMA can induce both Ca^{2+} influx and intracellular Ca^{2+} release, whereas activation of postjunctional α_2 -adrenoceptors in the CMCA appears to stimulate Ca^{2+} influx only. The presence of different α -adrenoceptor subtypes cannot by itself adequately explain the differences found between cerebral and mesenteric arteries in the sensitivity of the NA-induced contraction to Ca^{2+} deprivation and nifedipine. It is suggested that the mechanism through which NA induces contractions is related not only to the subtype of α -adrenoceptor stimulated by NA, but also to the anatomical origin of the vessel.

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Table 1. Relaxation produced by nifedipine of K^+ -contracted rat (RMCA) and cat (CMCA) middle cerebral arteries.

Artery	n	Relaxation	
		$-\log RC_{50}^a$	$R_{max} (\%)^b$
RMCA	7	8.41 ± 0.06	84 ± 2
CMCA	6	8.48 ± 0.05	87 ± 2

^a Logarithm of the nifedipine concentration (M) producing half maximum relaxation

^b Maximum relaxation

Table 2. Inhibition by nifedipine of the maximum response and the tonic component of the contractile response to 10 μ M noradrenaline in rat and cat middle cerebral (RMCA, CMCA) and mesenteric (RMA, CMA) arteries. Conditions as in fig. 6.

Artery	n	I _{max} (%) ^a		-log IC ₅₀ ^b		Tonic component of the NA response ^c
		Maximum to NA	Maximum response of the NA response ^c	Maximum response to NA	Maximum response of the NA response ^c	
RMCA	9	48 $\pm$ 5	77 $\pm$ 3	8.02 $\pm$ 0.06	8.02 $\pm$ 0.09	
RMA	8	25 $\pm$ 4	41 $\pm$ 6	7.38 $\pm$ 0.13	7.15 $\pm$ 0.10	
CMCA	6	86 $\pm$ 2	92 $\pm$ 2	8.65 $\pm$ 0.11	8.70 $\pm$ 0.13	
CMA	6	34 $\pm$ 3	37 $\pm$ 3	7.57 $\pm$ 0.07	7.58 $\pm$ 0.08	

^a Maximum inhibition produced by nifedipine.

^b Logarithm of the nifedipine concentration (M) producing half maximum inhibition.

^c The tonic component was defined as the amplitude of the contraction 5 min after application of NA.

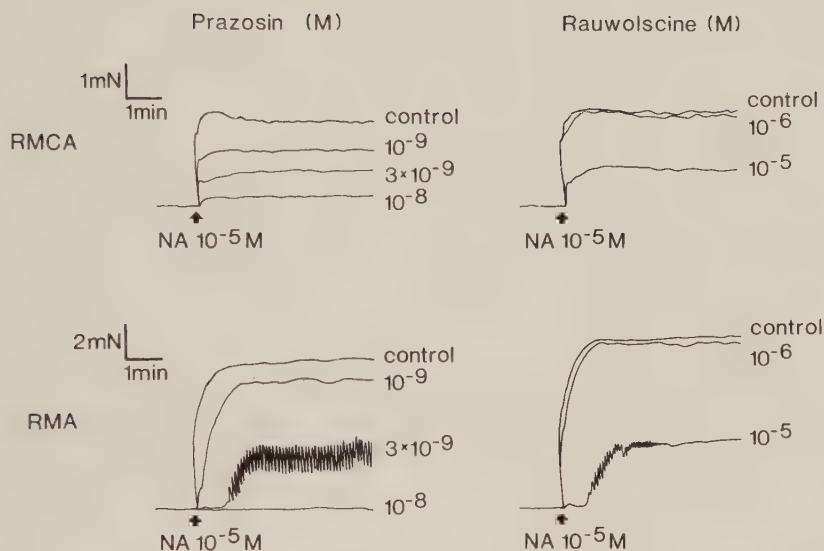


Fig.1. Superimposed tracings of contractions induced by noradrenaline (NA) in the presence of increasing concentrations of prazosin or rauwolscine in rat middle cerebral (RMCA) and mesenteric (RMA) arteries. The exposure time for each concentration of α -adrenoceptor antagonist was 15 to 20 min. Each series of tension curves was recorded from a separate arterial segment.

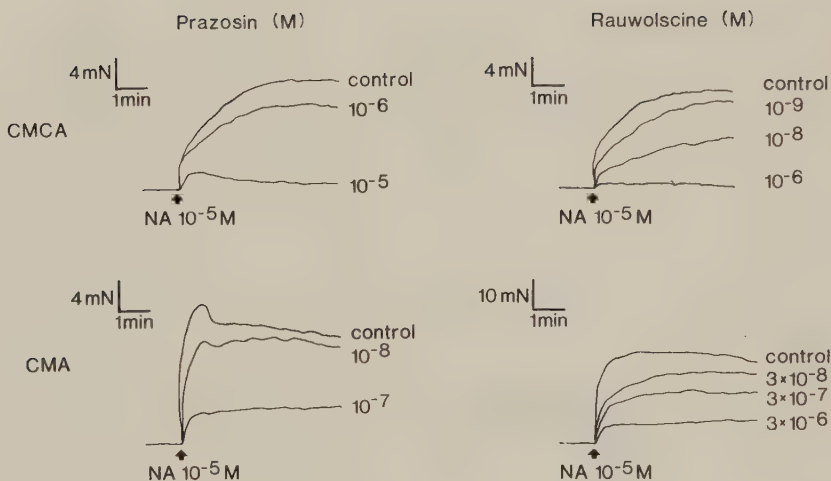


Fig. 2. Suppression of noradrenaline (NA)-induced contractions by increasing concentrations of prazosin and rauwolscine in cat middle cerebral (CMCA) and mesenteric (CMA) arteries. Experimental conditions as in Fig. 1.

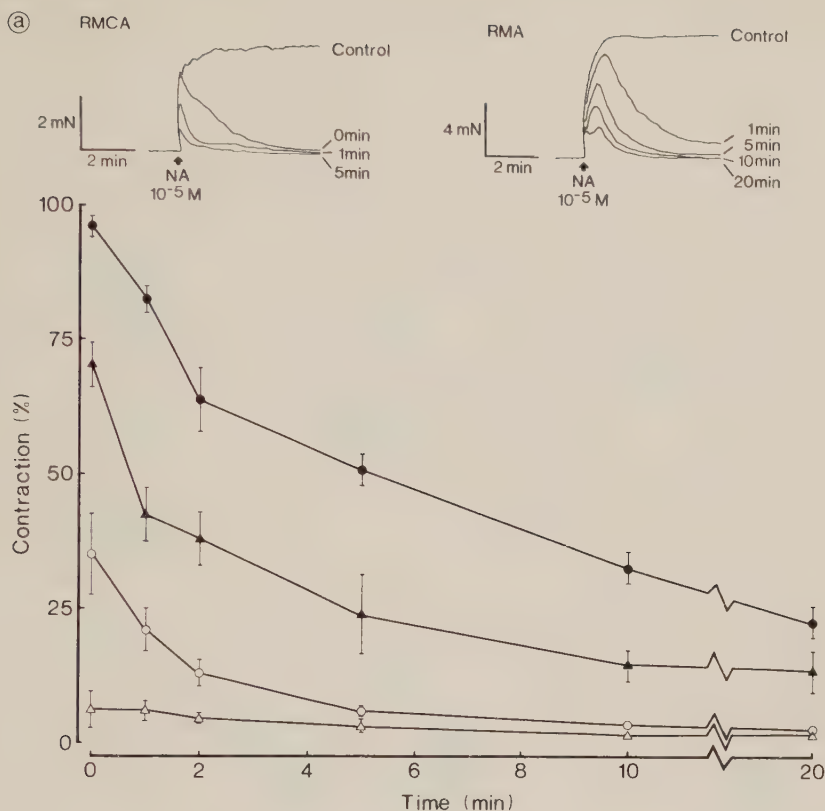


Fig. 3a. Effect of Ca^{2+} removal on the contractile response to 10 μ M noradrenaline (NA) in rat middle cerebral (RMCA; ▲, △) ($n=5-8$) and mesenteric (RMA; ●, ○) ($n=6-8$) arteries. The time indicated refers to the time period in Ca^{2+} -free medium before application of NA. In some experiments the RMCA and RMA were exposed to a Ca^{2+} -free NA solution without prior treatment in Ca^{2+} -free medium (shown at time "0 min"). The effects of Ca^{2+} omission on the maximum response (filled symbols) and on the tonic component (open symbols) of the NA-induced contraction were evaluated separately and expressed as a percentage of respective response in standard Krebs solution. The tonic component was defined as the amplitude of the contraction 5 min after application of NA. The arteries were allowed to recover in standard Krebs solution for at least 20 min following each exposure to Ca^{2+} -free medium. Upper section: Superimposed tension tracings from two individual arterial segments.

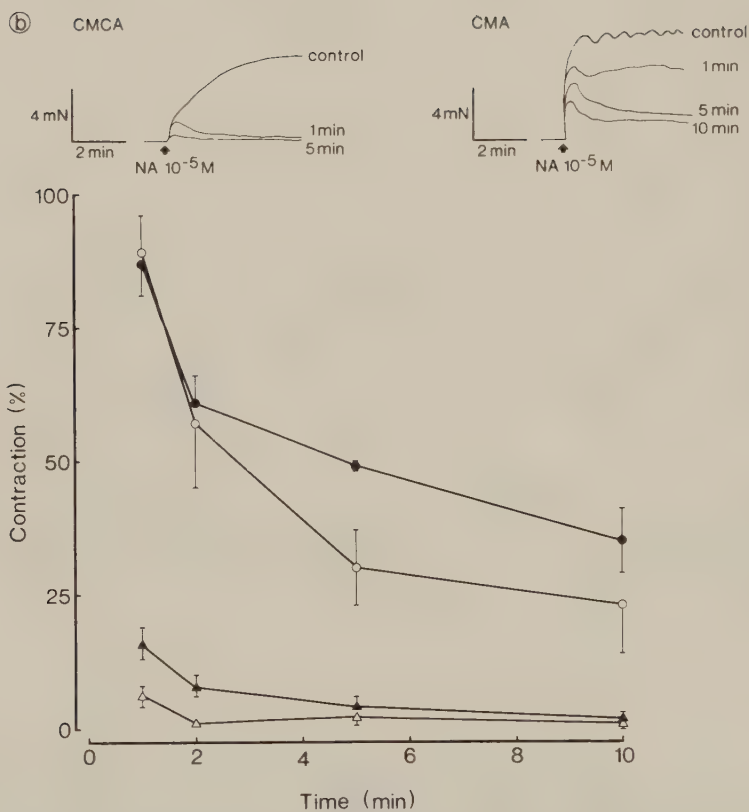


Fig. 3b. Effect of Ca^{2+} removal on the contractile response to noradrenaline (NA) in cat middle cerebral (CMCA; ▲, △) ($n=6$) and mesenteric (CMA; ●, ○) ($n=6$) arteries. For further explanations, see legend to Fig. 3a.

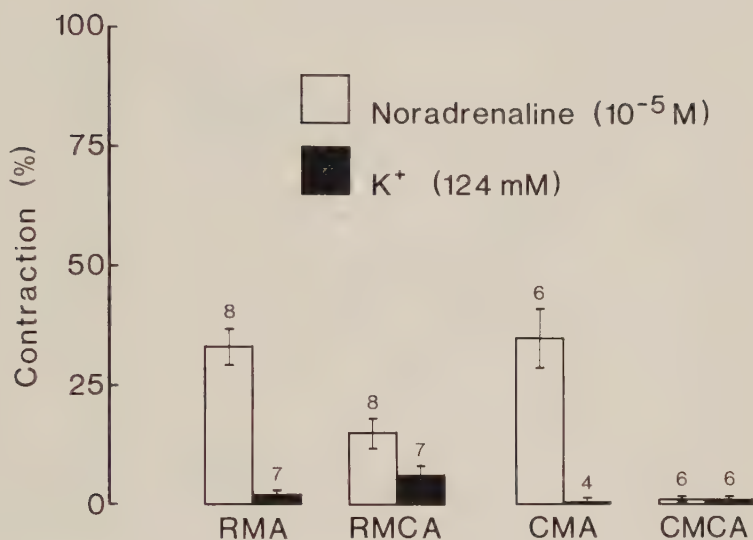


Fig. 4. Reduction of the maximum contractile responses to $10 \mu\text{M}$ noradrenaline (NA) and $124 \text{ mM } K^+$ following 10 min in Ca^{2+} -free medium. 100 % denotes the maximum contraction evoked by respective stimulant in standard Krebs solution. RMA, rat mesenteric artery; CMA, cat mesenteric artery; RMCA, rat middle cerebral artery; CMCA, cat middle cerebral artery. Figures above the bars indicate the number of vessels examined.

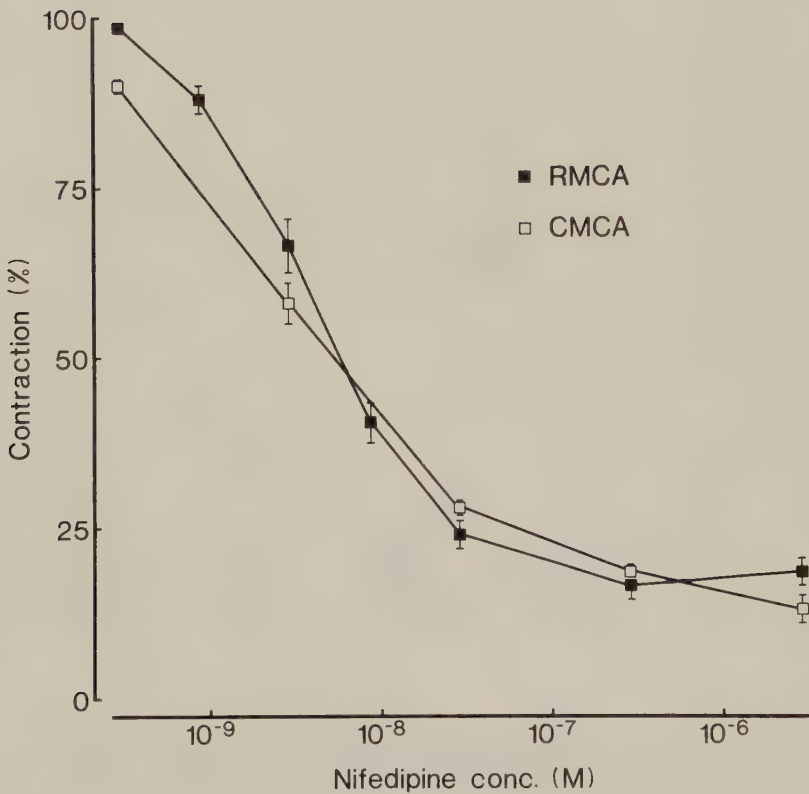


Fig. 5. Concentration-relaxation curves for nifedipine in K^+ -contracted rat (RMCA) ($n=7$) and cat (CMCA) ($n=6$) middle cerebral arteries. Contractions were induced by a high K^+ solution, containing 124 mM K^+ . After approximately 5 min in this medium, at a time when the tension had stabilized, nifedipine was added cumulatively. The tension was allowed to reach a plateau following each application of nifedipine; this usually required between 5 and 10 min.

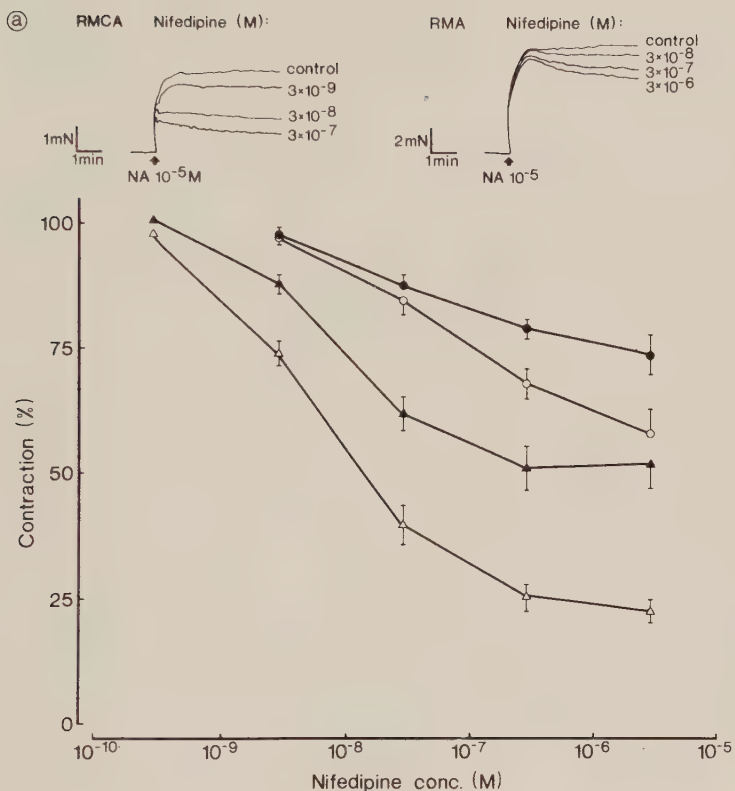


Fig. 6a. Inhibition of noradrenaline (NA)-induced contractions by increasing concentrations of nifedipine in rat middle cerebral (RMCA; ▲, △) ($n=7-9$) and mesenteric (RMA; ●, ○) ($n=6-9$) arteries. Each concentration of nifedipine was applied to the tissue 15 to 20 min before application of NA ($10 \mu\text{M}$). The inhibition produced by nifedipine was calculated in two ways: Filled symbols refer to the maximum response; open symbols refer to the amplitude of the contraction 5 min after application of NA (tonic component). 100 % denotes the maximum response or the tonic component of the NA-induced contraction before introduction of nifedipine. Upper section: Superimposed tension tracings from two individual arterial segments.

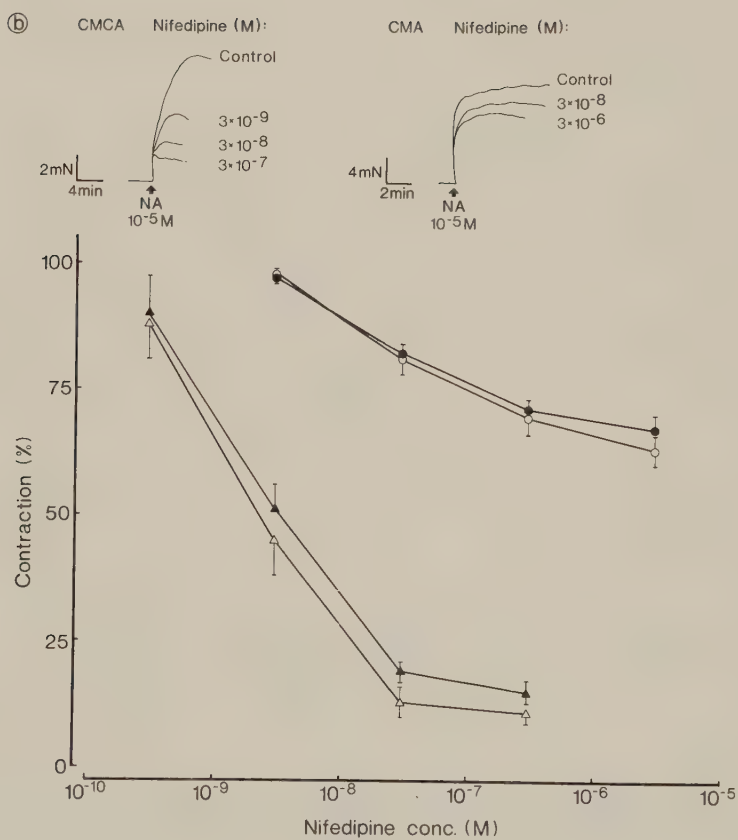


Fig. 6b. Inhibition of noradrenaline (NA)-induced contractions by increasing concentrations of nifedipine in cat middle cerebral (CMCA; ▲, △) ($n=5-7$) and mesenteric (CMA; ●, ○) ($n=7-8$) arteries. For further explanations, see legend to Fig. 6a.

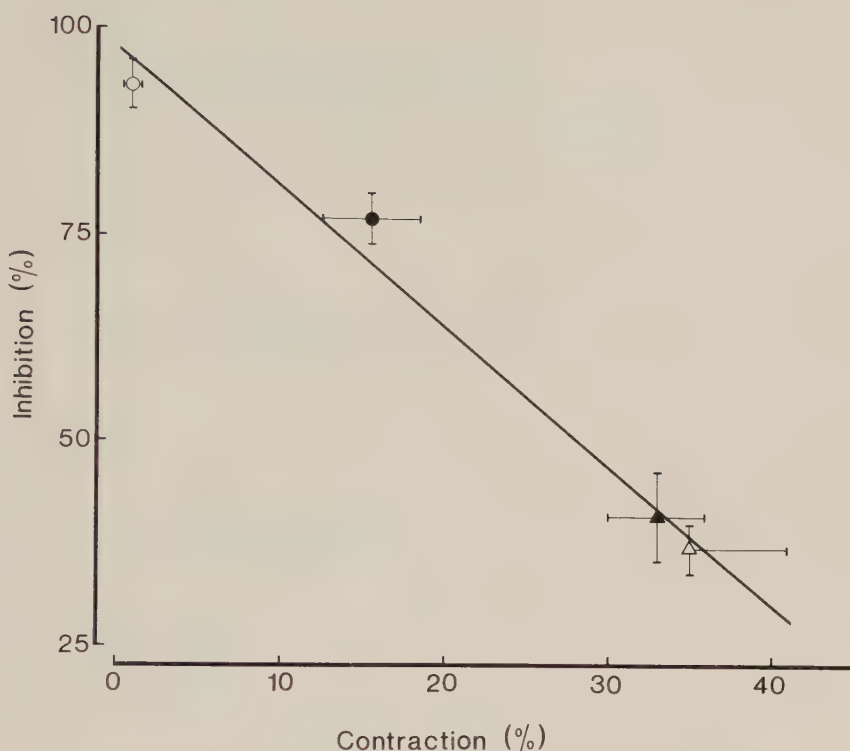


Fig. 7. Linear correlation ($r=-0.99$, $P < 0.01$) between the mean inhibition (ordinate) produced by $3 \mu\text{M}$ nifedipine of the tonic component of the contractile response to $10 \mu\text{M}$ noradrenaline (NA), and the mean amplitude of the contraction (abscissa) elicited by NA after 10 min in Ca^{2+} -free medium, as revealed by regression analysis. ●, rat middle cerebral artery; ▲, rat mesenteric artery; ○, cat middle cerebral artery; △, cat mesenteric artery. The tonic component of the NA-induced contraction was taken as the amplitude of the response 5 min after application of the agonist. The contraction induced by NA in Ca^{2+} -free medium was expressed as per cent of the corresponding control response in standard Krebs solution. Each mean value was based on 5 to 9 experiments. The time of exposure to nifedipine was approximately 15 min. The equation of the regression line is: $y = -1.7x + 98$.

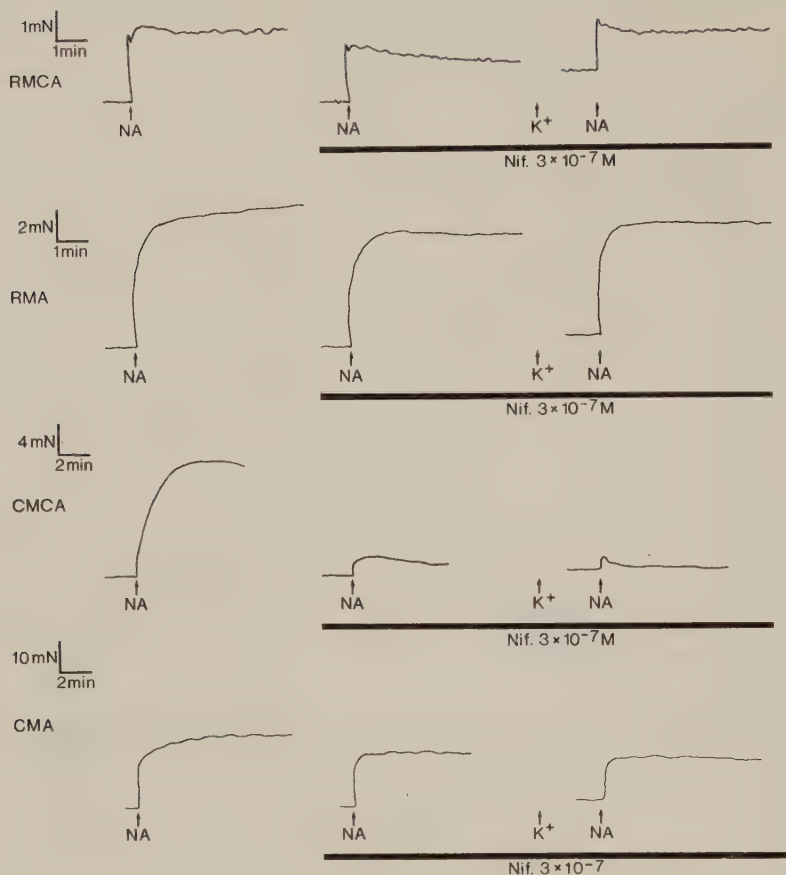


Fig. 8. Effect of prior K^+ depolarization (in the presence of $0.3 \mu\text{M}$ nifedipine) on the contractile response to $10 \mu\text{M}$ noradrenaline (NA) in rat and cat middle cerebral (RMCA, CMCA) and mesenteric (RMA, CMA) arteries. Results from four different experiments are shown. All vessel segments were treated identically. From the left to the right: **First arrow**, control contraction induced by NA in standard Krebs solution. **Second arrow**, response to NA in the presence of nifedipine (horizontal bars). Nifedipine (Nif.) was added 15 min before NA and then kept present throughout the experiment. **Third arrow**, exposure to a high K^+ solution, containing $124 \text{ mM } K^+$. This evoked a transient contraction (not shown), which stabilized slightly above the baseline tension after 5 to 10 min. **Fourth arrow**, application of NA 5 to 10 min after introduction of K^+ .

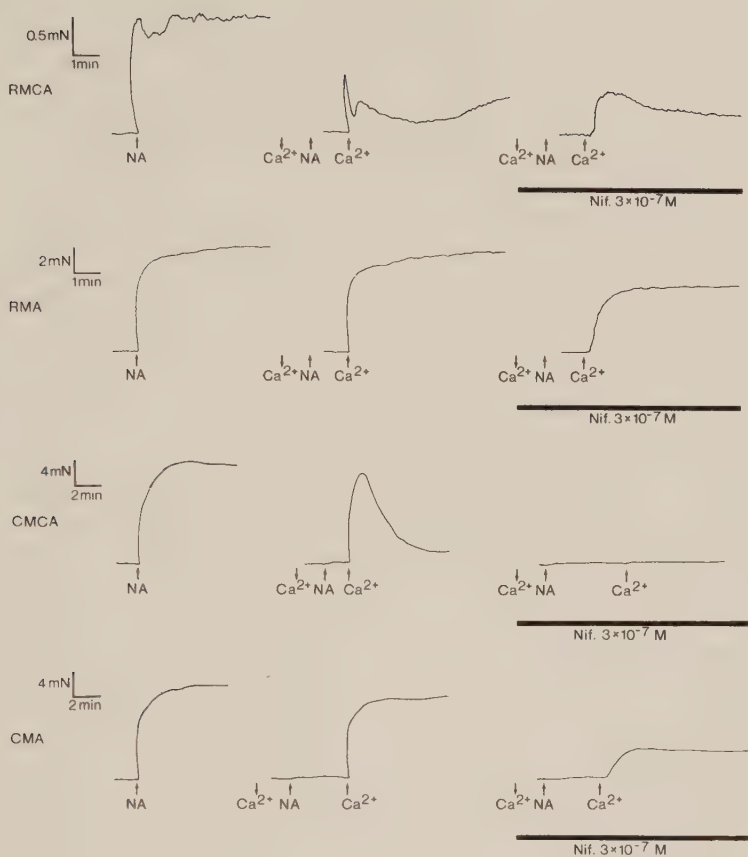


Fig. 9. Effect of nifedipine (Nif.) on Ca^{2+} -induced contractions in noradrenaline ($10 \mu\text{M}$)-exposed rat and cat middle cerebral (RMCA, CMCA) and mesenteric (RMA, CMA) arteries. The same experimental protocol was used in all experiments. From the left to the right: **First upward arrow**, control contraction elicited by noradrenaline (NA) in standard Krebs solution. **First downward arrow**, removal of Ca^{2+} by replacement with a Ca^{2+} -free Krebs solution ($10 \mu\text{M}$ EGTA). **Second upward arrow**, introduction of NA to the bath solution. This sequence was repeated until the response to NA in Ca^{2+} -free medium was reduced to a minimum. **Third upward arrow**, readdition of 1.5 mM Ca^{2+} to the bath solution. The Ca^{2+} -induced contraction was subsequently repeated a second time in the presence of nifedipine ($0.3 \mu\text{M}$). The exposure time for nifedipine was 15 to 20 min.

